FORMULATION AND EFFICACY OF LIPOSOME-ENCAPSULATED AZITHROMYCIN FOR PULMONARY INFECTION DUE TO *PSEUDOMONAS AERUGINOSA*

by

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Abstract

Cystic fibrosis is the most common life-threatening autosomal recessive disorder in Caucasians. Recurrent pulmonary infection and inflammation are the major risk factors associated with cystic fibrosis. Microbial infection with highly resistant pathogens such as *Burkholderia cenocepacia* and *Pseudomonas aeruginosa,* is principally associated with cystic fibrosis. The effective management of pulmonary infection in cystic fibrosis patients is not controllable due to the multidrug-resistant strains and potential side effects of antibiotics usage. Liposomal encapsulation of macrolide antibiotics such as azithromycin show increased drug concentrations at the site of infection, along with reduced toxic effects. In this thesis work, liposome-loaded azithromycin formulation was prepared by dehydration-rehydration vesicle method and related characterizations including cytotoxicity were identified. The effects of liposomal azithromycin on biofilm community, purified bacterial virulence factors were determined and motility studies were performed in clinical isolate of *P. aeruginosa*. We found that liposomal azithromycin reduced biofilm activity, virulence factors production and bacterial motility. The liposomal formulation confirmed interactions between liposomes and bacterial membranes besides insignificant hemolysis or A549 cell toxicity. The end results collectively indicate that liposomal drug delivery systems could be a promising model to enhance the efficacy of antibiotics against resistant bacterial strains in lung infections.

Keywords: Cystic fibrosis, Liposomes, Azithromycin.

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Dedication

I dedicate this thesis to my family, friends and well-wishers.

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List of Abbreviations

A549:	Human Lung Carcinoma Epithelial Cell Line
ABC:	ATP-Binding Cassette
AHL:	Acyl Homoserine Lactone
ANOVA:	Analysis of Variance
ATCC:	American Type Culture Collection
ATP:	Adenosine Triphosphate
Bcc:	Burkholderia cepacia Complex
$CaCl_2$:	Calcium Chloride
CAMH:	Cationic-Adjusted Mueller-Hinton
CBD:	Calgary Biofilm Device
Chol:	Cholesterol
CF:	Cystic Fibrosis
CFTR:	Cystic Fibrosis Transmembrane Conductance Regulator
CFU:	Colony Forming Unit
Cl- :	Chloride
CLSI:	Clinical and Laboratory Standards Institute
CPT:	Chest Physical Therapy
CYP3A4:	Cytochrome P-450 system
DDAB:	Didecyldimethylammonium bromide
DNA:	Deoxyribonucleic Acids
DCP:	Dicetyl phosphate

ΔF508:	Delta Phenylalanine Amino Acid at Codon 508
DLS:	Dynamic Light Scattering
DMPC:	1, 2-dimyristoyl-sn-glycero-3-phosphocholine
DMEM:	Dulbecco's Modified Eagle's Medium
DOPC:	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DPPC:	Dipalmitoylphosphatidylcholine
DPPG:	1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol
DPBS:	Dulbecco's Phosphate Buffered Saline
DRV:	Dehydration-Rehydration Vesicles
DSC:	Differential Scanning Calorimetry
DSPC:	1, 2-Distearoyl-sn-glycero-3-phosphocholine
DSPE:	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
DSPG:	1, 2-Distearoyl-sn-glycero-3-phosphoglycerol
EE:	Encapsulation Efficiency
EPC:	Egg-PC
EPS:	Extracellular Polymeric Substance
ER:	Endoplasmic Reticulum
FACS:	Fluorescence-Activated Cell Sorting
FBS:	Fetal Bovine Serum
Free AZM:	Free Azithromycin
HPLC:	High Performance Liquid Chromatography
HSPC:	Hydrogenated Soy PC
LAI:	Liposomal Amikacin for Inhalation

LB broth:	Luria-Bertani broth
Lipo-AZM:	Liposomal Azithromycin
LPS:	Lipopolysaccharides
LUV:	Large Unilamellar Vesicles
MAC:	Mycobacterium Avium Complex
MBC:	Minimum Bactericidal Concentrations
MBEC:	Minimum Biofilm Eradication Concentration
MIC:	Minimum Inhibitory Concentration
MLV:	Multilamellar Liposomes Vesicles
mRNA:	Messenger Ribonucleic Acid
MRSA:	Methicillin-resistant Staphylococcus aureus
MSD:	Membrane Spanning Domain
MTT:	Methyl Thiazol Tetrazolium
Na+:	Sodium
NAC:	N-Acetly-L-Cystiene
NBD:	Nucleotide-Binding Domain
NSAID's:	Non-Steroid Anti-Inflammatory Drugs
NTM:	Non-Tuberculosis Mycobacteria
OM:	Outer membrane
PBS:	Phosphate-Buffered Saline
PC:	Phosphatidylcholines
PE:	Phosphatidylethanolamines
PI:	Polydispersity Index

PS:	Phosphatidylserine
PTC:	Premature terminating codons
P-NMR:	Phosphorus nuclear magnetic resonance
QS:	Quorum sensing
R:	Regulatory domain
REV:	Reverse-phase Evaporation Vesicles
RPMI:	Roswell park memorial institute medium
RNA:	Ribonucleic Acids
SAXS:	Small angle X-ray scattering
SEM:	Standard Errors of the Mean
Sub-MIC:	Sub-Minimum Inhibitory Concentration
SUV:	Small Unilamellar Vesicle
TEM:	Transmission Electron Microscopy
TSST-1:	Toxic Shock Syndrome Toxin-1

Chapter 1

1 Introduction

1.1 Cystic Fibrosis (CF)

CF is a common lethal autosomal recessive disorder, which particularly affects Caucasians in the proportion of one in 2,500 persons.[1,2] CF is a multi-organ disease primarily affecting lungs, pancreas, and sweat glands. Up to some extent, CF also affects organs such as the liver, gastrointestinal tract and reproductive organs.[3,4] Out of every systems, the most affected is the pulmonary system. Consequently, pulmonary injury is the major clinical manifestation and the key reason for CF affected patient's death.[5] The underlying reason for CF pathophysiology is cause of CF is mutations in the Cystic Fibrosis Transmembrane Conductance (CFTR) gene.[6,7]

1.1.1 Pathophysiology

The defective CF gene causes malfunction in CFTR protein, and subsequent to this alteration, conductance of sodium and chloride on plasma membrane of the epithelial cell get deviated.[8] The consequences are depletion of airway surface fluid and abnormal glandular secretions. The disease symptoms include, the mucus thickens and small airways are blocked (Diagram 1).[9] The decreased intake of sodium and chloride ions, removal of bacteria by defective epithelial ciliary function bring about a stagnant sputum in the lungs, which also intensifies infection and inflammation.[10] Followed by a condition known as bronchiectasis, the bronchi dilate and weaken, causing large airway obstruction.[11] The continuous vicious cycle of airway obstruction is recurrent bacterial infection and inflammation that prompts a diminished lung function and respiratory failure, a common cause of CF mortality.[12]

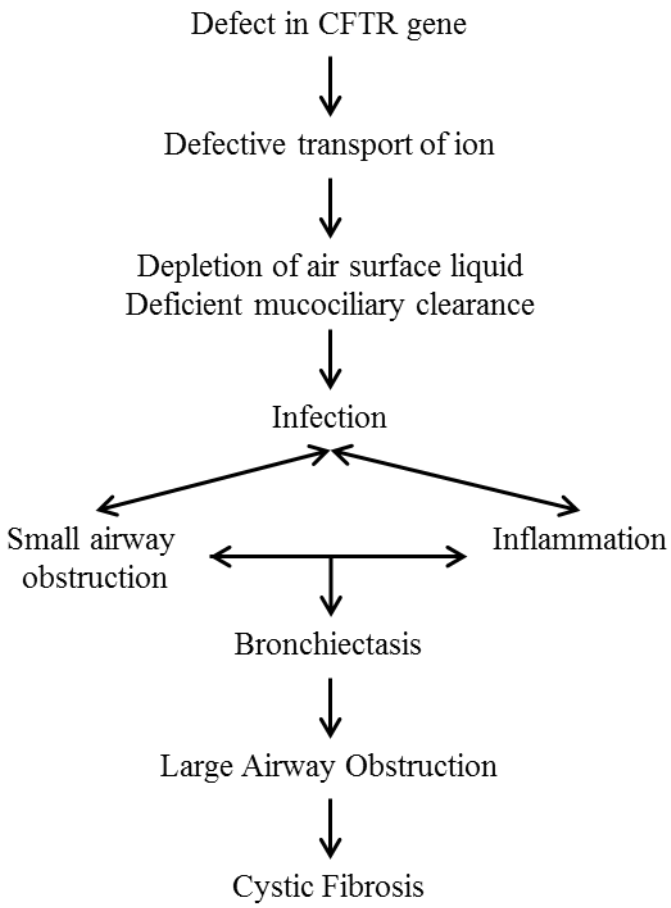


Diagram 1: Pathophysiology of Cystic Fibrosis Syndrome

1.1.2 Cystic Fibrosis Transmembrane Conductance (CFTR)

Approximately, 2000 CFTR mutations have been recognized and classified into 6 types based on their effects.[13] The deletion of phenylalanine in the amino acid position at Δ508 is the most common mutation in CF community.[14] The CFTR chloride channel is associated with the process of releasing Adenosine triphosphate (ATP) and coordinating other ion transport channels.[15] CFTR is mainly circulated in respiratory epithelial cells, as well as the biliary tract, pancreas, and genitourinary system.[16]

1.1.2.1 Structure of CFTR protein

The CFTR gene is based on the single arm of chromosome 7 and spans 250-kb of DNA.[17,18] CFTR belongs to an Adenosine triphosphate (ATP)-binding cassette family of proteins and is a member of an ATP Binding Cassette (ABC) family.[19]

The CFTR consists of 7 domains (Diagram 2):

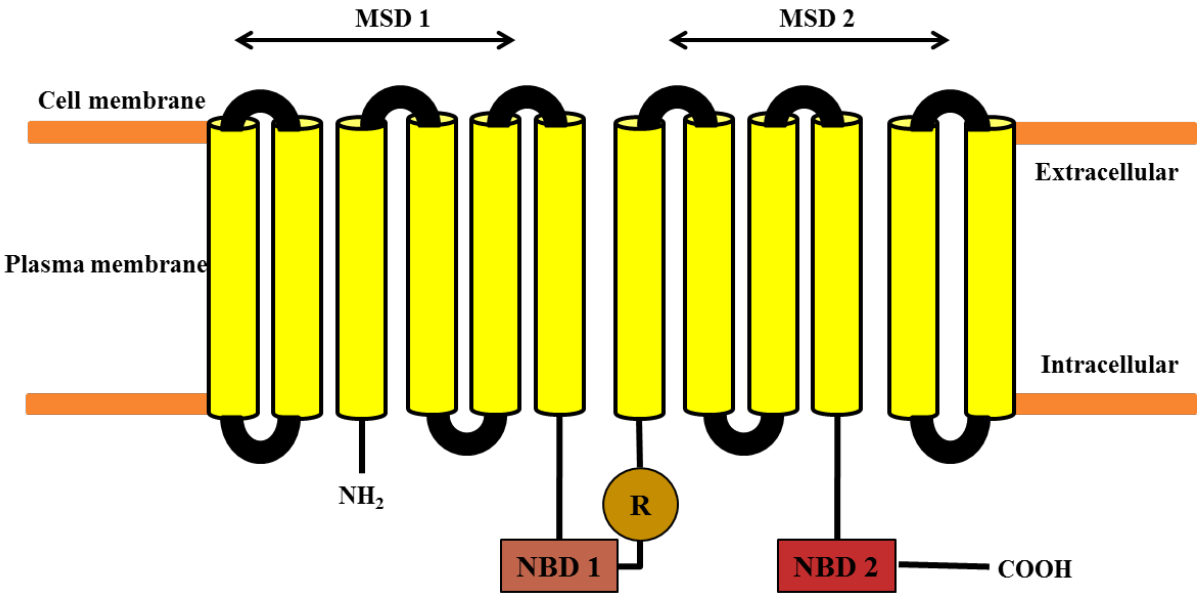
1) Intracellular amino and carboxyl terminal domains

2) Two 6-segment Membrane-Spanning Domains (MSD)

3) Regulatory (R) - domain

4) Two Nucleotide-Binding Domains (NBD)

The CFTR structure consists of sequential repeats of ABC pattern and is separated by a regulatory R-domain.[20] The ABC pattern includes MSD, which is composed of six transmembrane stretches of amino acids followed by the NBD.[21] The function of NBD is to bind and hydrolyze ATP to conduct chloride channel function. NBD-1 opens the chloride channel and NBD-2 closes the channel by ATP hydrolysis. This function is coordinated by phosphorylation of serine residues located in the R domain.[22]



MSD: Membrane Spanning Domain, NBD: Nucleotide Binding Domain
R: Regulatory Domain

Diagram 2: Structure of CFTR protein

1.1.2.2 Types of CFTR mutations

CFTR mutations are classified into six types based on mechanism of inducing disease and functions as shown in Diagram 3.[23] The class I mutations, also known as stop mutations, regulates protein production and compromise approximately 10% of the total CF cases.[16,24] Class II mutations consist of the most common ΔF508 mutations with typical CF symptoms.[16] Class III (gating) mutations are rare and function in decreasing channel activation, which results in closure of the channels.[25] Class IV mutations decrease ion conductance while,[26] Class V mutations cause deformity in the CFTR molecules with reduced performance.[27]Lastly, Class VI mutations result in unstable proteins with an accelerated degradation. Collectively, these mutations affect the CFTR regulatory function on cell surfaces.[16]

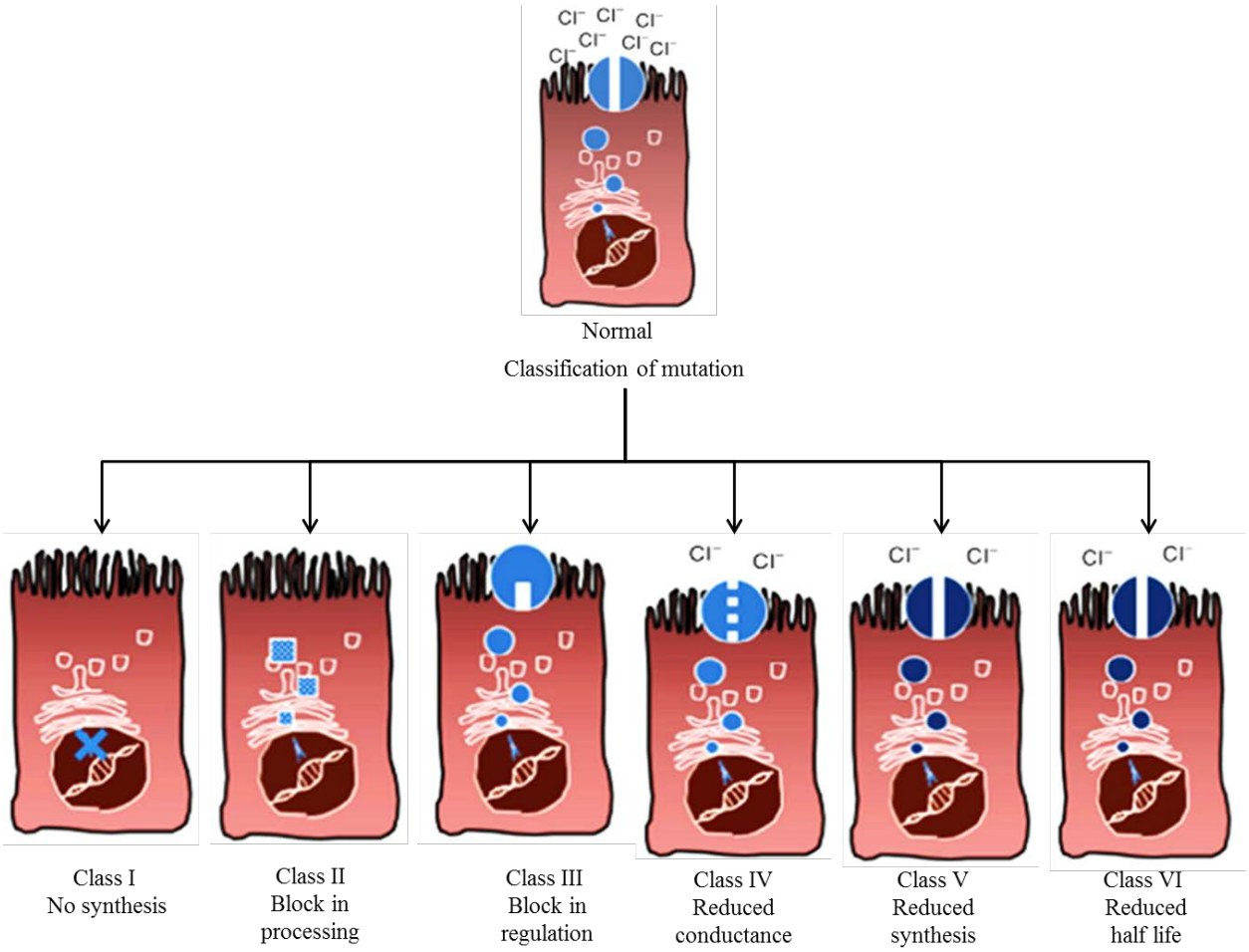


Diagram 3: Types of CFTR Mutations

(Modified from Esther et al 2014)

1.1.2.3 Function of CFTR in lungs

In CF, lung disease is the main cause of morbidity and mortality, though it affects other organs as well. The gastrointestinal tract, liver, pancreas, and sweat glands equally contribute to the progression of the disease.[28] The normal function of CFTR in the lungs is to regulate water and salt levels across the epithelial linings. In dysfunctional CFTR, hyper activity of epithelial sodium channels (ENaC) and failure of chloride ion to transport Cl^- to the lumen result in the depletion of airway surface fluid (Diagram 4).[29] Alterations in ciliary beating and accumulation

of more mucus on the airway surface, makes the lumen a favorable niche for microbial growth.[30] Gram-positive *Staphylococci* species, and Gram-negative *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* species are the major opportunistic pathogens implicated in CF pulmonary infection.[31,32]

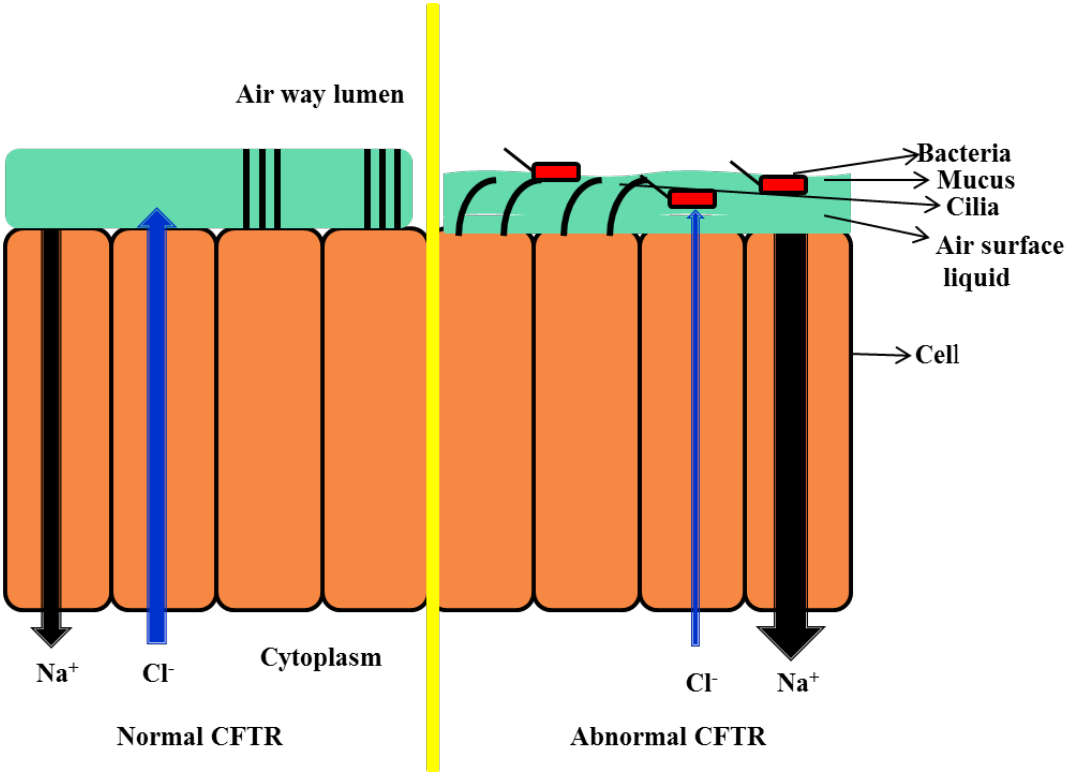


Diagram 4: Function of Normal and Abnormal CFTR

1.1.3 Comparison of normal and cystic fibrosis airways

The normal airways are covered by two layers of mucus. The first is viscous fluid on the upper layer, and the second is periciliary fluid on the lower layer (Diagram 5).[33] The mucus layers are needed for the continuous beating of epithelial cell cilia, which moves the mucus in a single direction flow towards the esophagus. During this process entrapped microorganisms in the mucus are carried away along with the flow. In the CF airways, the modifications in the mucus layers by abnormal ion secretion result in a uniformly sticky fluid that traps bacteria.[34]

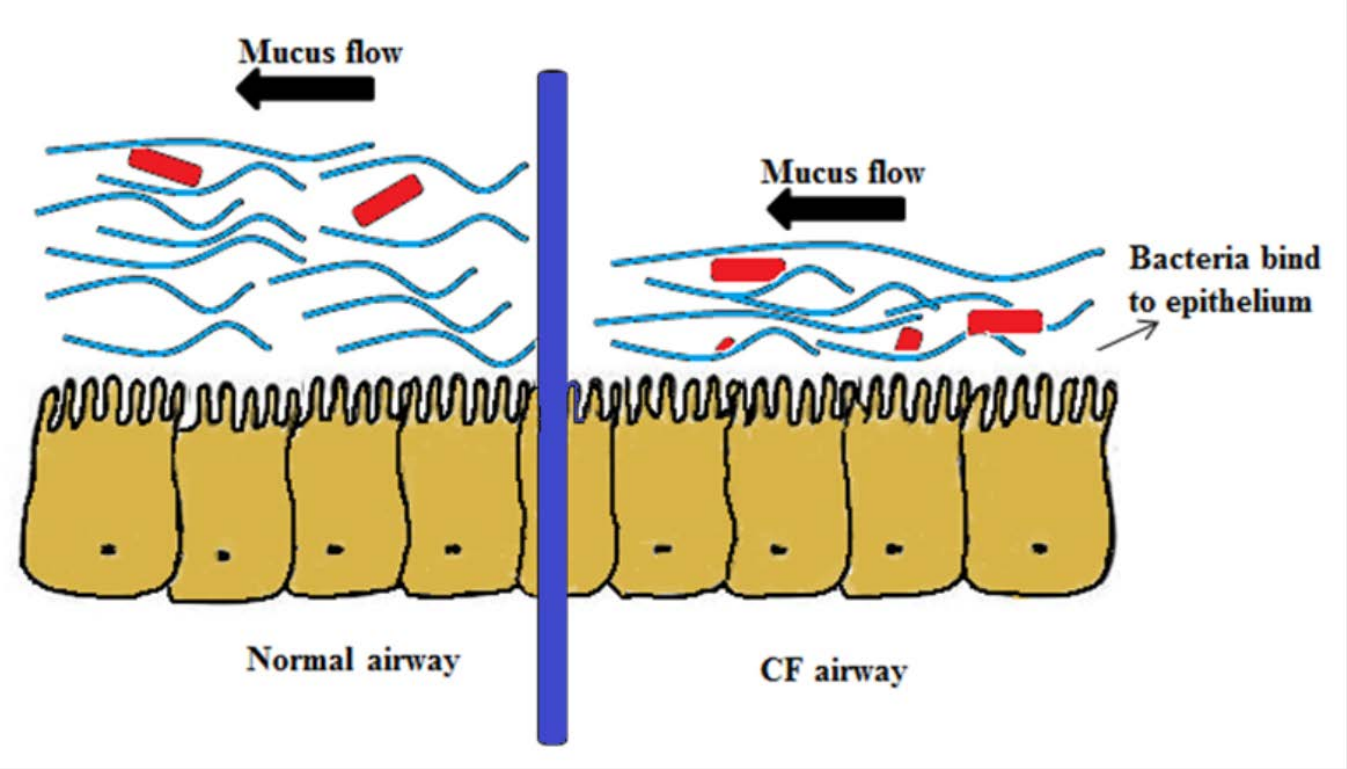


Diagram 5: Comparison of Mucociliary Mechanism in Normal and CF Airways

1.2 Microbiology of CF airways

In a healthy individual's lungs, the colonized bacteria are eradicated by the immune response of the body. However, the CF patient's immune system overwhelmed by the bacterial colonization is unable to disperse the foreign bacteria residing in the lungs.[35] In CF airways, infection is a conventional cohort of different microbial species such as *S. aureus*, *H. influenzae*, *B. cepacia complex* and *P. aeruginosa.*[36,37]

S. aureus is non-motile, Gram-positive bacteria, facultative anaerobic microorganism mainly accountable for nosocomial and community-acquired infections.[38] *S. aureus* are mainly responsible for causing pulmonary infections in CF patients.[39] *H. influenza* is Gram-negative bacteria accountable for causing respiratory infections in both children and adults. [40] *H. influenza*

causes inflammation and damage to the airway.[40] This damaged airway surface creates favorable conditions for colonization of *P. aeruginosa* in CF patients.[41] *S. aureus* and *H. influenza* are the primary bacterial pathogens infecting the lungs of pediatric CF patients.[42]

B. cenocepacia and *P. aeruginosa* are the major opportunistic Gram-negative pathogens associated with the infection, inflammation and mortality in adult CF patients.[43,44] Gram negative *P. aeruginosa* and *B. cenocepacia* co-exist in the lungs of CF individuals and also form mixed biofilms during the infection process.[35] In most CF cases, the *P. aeruginosa* colonization is followed by *B. cenocepacia* in the lungs and inhabits similar environmental conditions.[45] It has been seen that the extracellular products of *P. aeruginosa* favor the settlement of *B. cenocepacia* by modifying the epithelial lining of the lungs in CF individuals.[46] The interspecies cell-to-cell communication between *P. aeruginosa* and *B. cenocepacia* plays an important role in the production of virulence factors, biofilm development, and pathogenesis of CF.[47]

The *Burkholderia cepacia* complex (BCC) are a group of related species (>20 species) mainly capable of causing respiratory infections.[48] *B. cenocepacia* is the most prevalent bacterial pathogen, affecting 50% to 80% of CF population.[49] This Gram-negative, rod-shape microbe mainly found in soil, plants, crops and any moist environment.[50] *B. cenocepacia* has been reported in many CF cases for direct transmission between CF individuals and associated with increased mortality.[51,52] *B. cepacia* complex species exhibited resistance to different antimicrobial agents as well.[53]

P. aeruginosa is the most opportunistic human pathogen and a major contributor of pulmonary exacerbation in the CF population. It is a Gram-negative bacillus affecting the lower respiratory tract of the lungs, and is associated with higher morbidity and mortality compared to

other CF causing pathogens.[54,55] *P. aeruginosa* is a ubiquitous micro-organism mainly found on plant surfaces, water, soil, and hospital enviornment.[56] It is the most prevalent respiratory bacterial pathogen in CF individuals as well as individuals with compromised immune system.[57] It can acquired by 70% - 80 % of adolescence CF patients.[58] *P. aeruginosa* colonization in the lungs is initiated by its adhesiveness with the help of epithelial cell receptors called asialogangloside-1 (GM1).[59] *P. aeruginosa* is known as a 'superbug' because of its inherent resistance to antibiotics attributed to various resistance mechanisms including outer membrane impermeability, biofilm formation, drug inactivation and efflux pumps.[60]

1.2.1 Biofilm formation

A biofilm is a structured and organized syndicate of bacterial cells implanted in self-produced polymer matrix attaching themselves to the living or abiotic surfaces (Diagram 6).[61,62] The matrix is made up of lipids, proteins, polysaccharides and extracellular DNA.[63] Bacterial biofilms are resistant to disinfectants, antimicrobial drugs and components of the immune system.[62] The bacteria in biofilm cause chronic bacterial infections leading to inflammation and continuous tissue damage.[64] The formation of biofilm undergoes several steps to form a mature biofilm, which include primary attachment to the surface, microfilm formation, mushroom shape formation of biofilm, and a detachment stage to release motile bacteria.[65] Type IV pili and flagella of *P. aeruginosa* are necessary for the formation of microcolonies and motility.[66] Quorum sensing signaling encoded genes *rhl* and *rhlab* are involved in the formation of mushroom-shaped biofilm structure.[67]

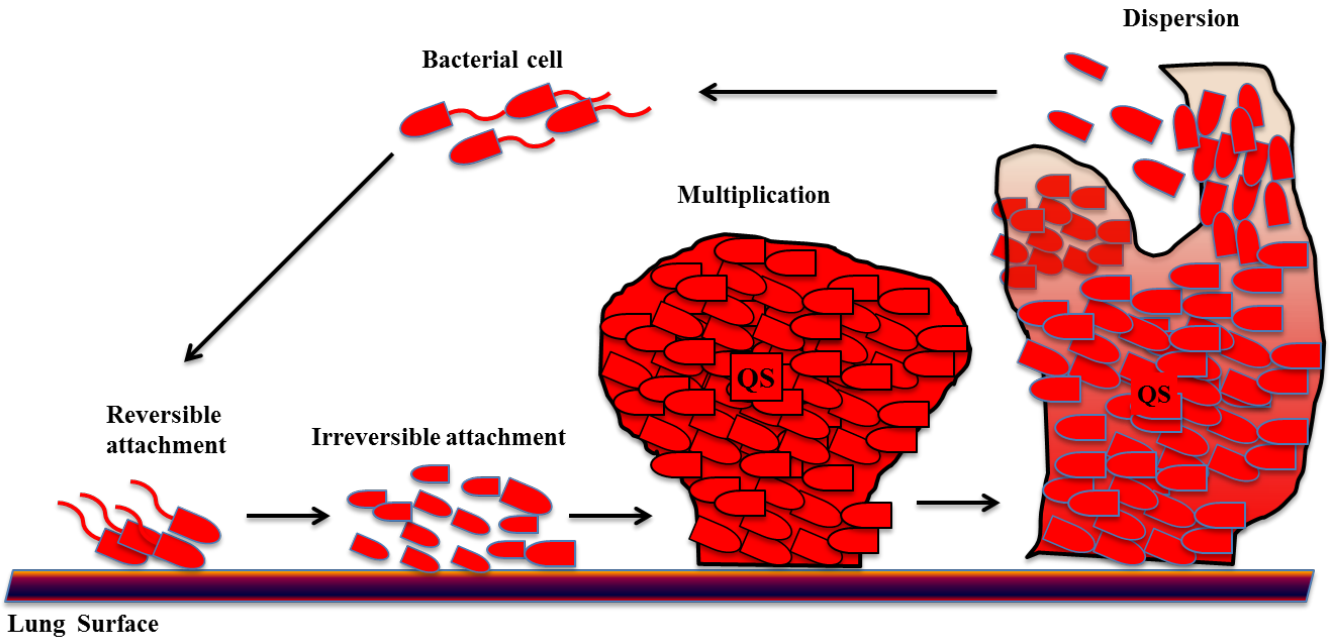


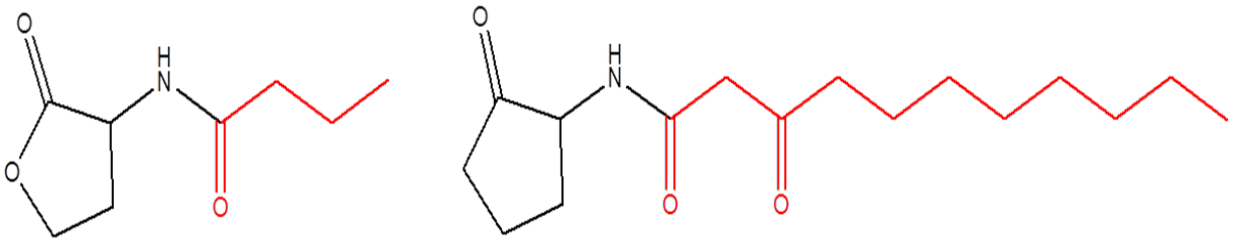
Diagram 6: Stages of Biofilm Formation

The production of matured biofilm by *P. aeruginosa* takes approximately 5-7 days.[62] The cycle of biofilm formation results in spreading planktonic bacteria to initiate new biofilm formation at different sites.[68] The formation of biofilms can be prevented by early aggressive antibiotic therapy followed by ongoing suppressive antibiotic therapy.[62,64]

1.2.2 Quorum Sensing

P. aeruginosa utilizes a distinct mechanism called Quorum Sensing (QS) which is cell-cell communication through signaling molecules produced in response to bacterial density.[69] The intercellular communication among *P. aeruginosa* is induced by bacterial products which are able to diffuse from one cell to another cell.[70] Quorum Sensing plays a significant role in regulating 10% of genes in the genome of *P. aeruginosa.*[71] Quorum sensing mechanism of *P. aeruginosa* bacteria produces small molecules called autoinducers for the regulation of gene expression. *P. aeruginosa* the genes *LasI* and *LasR* are responsible for sensing cell density and

production of other regulated genes.[30] The organism consists of two autoinducers (Diagram 7), N-butyryl-L-Homoserine lactone (C_4-HSL) and N-3-oxo-dodecanoyl-L-Homoserine lactone (3O-C_{12}-HSL) involved in exoproducts synthesis.[72,73] QS signaling molecules regulate gene expression that controls production of different virulence factors and formation of biofilm, thus contributing to *P. aeruginosa's* tolerance for antimicrobial chemotherapy.[74,75]



(a) N-butyryl-L-Homoserine lactone (C_4-HSL)

(b) N-3-oxo-dodecanoyl-L-Homoserine lactone (3O-C_{12}-HSL)

Diagram 7: Quorum Sensing Signaling Molecules

1.2.3 Virulence Factors

P. aeruginosa releases different virulence factors such as rhamnolipids, pyocyanin, pyoverdine, lipase, chitinase, elastase and proteases, which can cause tissue damage, proteolysis imbalance and immunomodulation.[76,77] Virulence factors released by *P. aeruginosa* provide favorable conditions for the settlement of bacteria and help in bypassing host immune responses.[78] Rhamnolipids are microbial glycolipids and biosurfuctants favors *P. aeruginosa* in bacterial cell motility and formation of biofilm.[79] Pyocyanin acts as both virulence factor and QS signaling molecule of *P. aeruginosa*, plays an important role in damaging pulmonary tissues.[80] Pyoverdine is an iron-chelating molecule (siderophore) of *P. aeruginosa* helps in the formation of flat biofilms and induces motility.[81] *P. aeruginosa* produces hemolytic enzyme phospholipase C causes extensive tissue damage and inflammation during CF lung infections.[82] After cellulose,

chitin is the most abundant polysaccharide found in nature and mainly on the bacterial cell walls. Chitinase possess mucolytic properties and are able to cause inflammatory (Type 2), allergic responses and remodeling of tissues.[83] The elastase of *P. aeruginosa* is encoded by the *Las*B gene of Quorum sensing regulating genes, which mainly leads to the destruction of respiratory epithelium and degradation of different plasma proteins.[84] Proteases (*LasA* elastase, *LasB* elastase and Alkaline proteases) are encoded by the *LasA* gene and play an important role in the disease progression of CF by damaging host tissue and remodeling tissues in the human airways.[85]

1.2.4 Antimicrobial resistance of *P. aeruginosa*

P. aeruginosa is a major opportunistic pathogen in the cause of CF population and its dissemination is very challenging to control because of its various multidrug-resistance mechanisms. The main mechanisms (Diagram 8) involved in the multidrug-resistance are bacterial biofilms, drug efflux systems, outer membrane permeability and enzymatic inactivation of the drugs.[86]

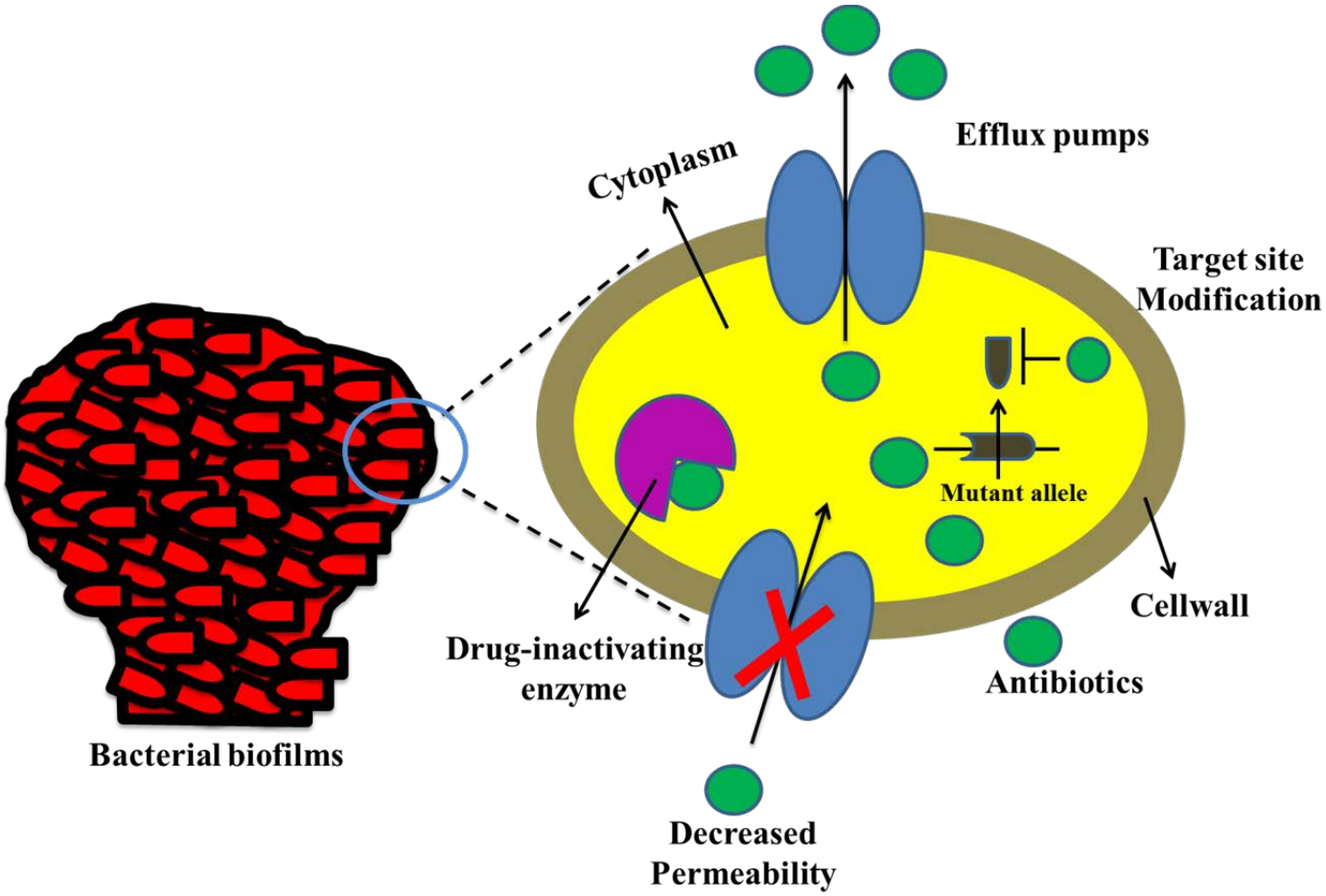


Diagram 8: Mechanisms of Multidrug-Resistance

1.2.4.1 Bacterial biofilms

Biofilms are embedded in the Extracellular Polymeric Substance (EPS) consists of polysaccharides and proteins that provide extra protection to the community.[87] The EPS matrix prevents antimicrobial entry by anion exchange.[88] The mutations in *mucA* gene with excess production of alginate provide further protection to bacterial biofilms.[89]

1.2.4.2 Efflux pumps

Efflux pumps are the major mechanism of the *P. aeruginosa* resistance to macrolide antibiotics. They prevent, through expulsion, drugs intake in to bacteria. The multidrug efflux

system of *P. aeruginosa* is comprised of three proteins, which are required for the effective removal of antibiotics from the bacteria.[90] The three components of the multidrug efflux system include joining protein (MexA), outer membrane porin M (OprM) and energy-dependent situated in the cytoplasmic membrane of the bacteria (MexB).[91] MexAB-OprM, MexCD-OprJ, and MexEF-OprN are the three components of the *P. aeruginosa* multidrug efflux system.[92] The efflux pumps MexAB-OprM and MexXY-OprM contribute to the intrinsic multidrug resistance, while hyper expression of MexAB-OprM, MexXY-OprM, and MexCD-OprJ provides acquired multidrug resistance.[93] The major mechanism of macrolide resistance in *P. aeruginosa* biofilms is due to hyper expression of MexCD-OprJ efflux pump, which plays an important role in the antibiotic resistance.[94] The MexCD-OprJ efflux pump is mainly expressed due to mutations in gene NfxB, which results in the increase of efflux genes and encodes a repressor of MexCD-OprJ efflux pump expression.[95]

1.2.4.3 Outer membrane permeability

The reduced permeability or uptake is one of the resistance mechanisms involved in the multi-drug resistance to *P. aeruginosa.* The outer membrane permeability in Gram-negative bacteria such as *P. aeruginosa* is the major barrier for both hydrophilic and hydrophobic drugs.[96] The main characteristic feature of Gram-negative bacteria like *P. aeruginosa* are an outer lipopolysaccharides (LPS) layer and inner phospholipids layer.[97] Cell membrane of *P. aeruginosa* contains porin proteins like OprD, which acts as passage way for various antimicrobial drugs or small chemical entities.[98] The reduced outer membrane (OM) permeability and presence of multi-drug efflux systems in *P. aeruginosa* work together, result in reduced antimicrobial drugs uptake.[99]

1.2.4.4 Enzymatic inactivation

The bacterium also exhibits multidrug-resistance as it comprises unique characteristics of inactivating enzymes and decreases the antibiotic activity of macrolides.[100] The group transferases is the class of resistant enzymes that inactivates the macrolide antibiotics by chemical substitution.[86] The modifications in antibiotics done by group transferases alter structural modifications (features) and impair the target site binding. The chemical strategies required for this process are *O*-acylation, N-acylation, *O*-phosphorylation, *O*-ribolysation, *O*-nucleotidylation and transfer of thiols.[86] Hydrolysis also causes antibiotic inactivation by targeting and cleaving the amides and esters chemical bonds prior to reaching target in bacteria. The target-site modification by methylation or mutation also prevents the binding of the antibiotic to its ribosomal target.[95]

1.3 Management of CF

1.3.1 Chest physical therapy

Chest Physical Therapy (CPT), also known as postural drainage and percussion, is the most widely used treatment in CF individuals. It is used for by clearing mucus from the airways of the CF lungs.[101] Excess mucus is removed from the CF lungs by using mechanical devices or different physiotherapy techniques.[102] The major drawbacks of CPT are that it is time-consuming, uncomfortable, and unpleasant to CF patients.

1.3.2 Lung transplantation

Lung transplantation is a major life-saving form of therapy for respiratory failure in CF patients associated with high-risks.[103] Lung transplantation in CF patients can increase significant survival rates and improve quality of life.[104]

1.3.3 Mucus thinning drugs

Mucus thinning drugs, also known as mucolytics, are designed to modify the biophysical properties of the mucus and reduces mucus elasticity and viscosity.[105] Depending upon the mode of action, mucolytics are classified as classic mucolytics, peptide mucolytics and non-destructive mucolytics.[106] The most widely used mucolytics are N-acetly L-cystiene (NAC), which acts by disrupting disulfide bonds of mucoproteins and dornase alfa by breaking down DNA enzymatically in the mucus.[107,108]

1.3.4 Bronchodilator therapy

Bronchodilator therapy is one of the most frequently used treatments for CF patients. Bronchodilators act by relaxing the smooth muscles of the airways and preventing bronchospasm in CF patients.[109] Bronchodilators are available in short-acting and long-acting medications and can be administered by dry powder inhaler, nebulizer and aerosol forms.[110] The short-acting drug salbutamol was widely used prior to CPT, which helps to open airway passages and drain mucus from the lungs of CF patients.[111] The long-acting drugs such as salmeterol and formoterol are used for the treatment of bronchoconstriction and have shown improved lung function in CF patients.[112]

1.3.5 Anti-inflammatory therapy

Various kinds of anti-inflammatory drugs are used in the treatment of CF to reduce the effects of inflammation in airway passages and lungs.[113] Higher levels of inflammatory responses like cytokines, neutrophils and chemokines are primarily associated with lung infection in CF.[114] The increased rate of inflammation results in destruction of the lung parenchyma and alterations in the mucociliary movements.[115] The oral corticosteroids such as prednisone are rarely prescribed for the treatment of CF for short term because of their potential side effects.[116] Inhaled corticosteroids such as beclomethasone and fluticasone deliver the drug directly to the lungs, thus causing less side effects in comparison to oral corticosteroids.[117] Non-steroid anti-inflammatory (NSAIDs) drugs such as ibuprofen are widely used in the treatment of CF as they possess major potential clinical benefits despite side effects like gastrointestinal hemorrhage.[118] The macrolide antibiotic azithromycin possesses both anti-inflammatory and immunomodulatory effects and has also shows an improved outcome in CF patients for extended usage.[119]

1.3.6 Antibiotic therapy

A wide range of antibiotics such as aminoglycosides, β-lactams, fluoroquinolones and macrolides are extensively used to control inflammation and Pseudomonas infections.[120,121] The goal of antibiotic therapy is to improve the quality of life for CF patients, and to decrease symptoms through reducing susceptibility to microbial infections.[122,123] The combination therapy of at least two antibiotics is more effective against various resistant strains and delayed resistance development compared to monotherapy. Aminoglycosides are common to use for respiratory infections however, side effects are associated thus constraint their clinical application and uses.

[124] β-lactams are broad range of spectrum of antibiotics consisting of β-lactam ring in their structure and their usage related with allergic reactions in CF patients. Fluoroquinolones are broad spectrum antibiotics used in the treatment of respiratory and urinary tract infections, however side effects like hepatotoxicity is associated with their usage. Alternatively, for pseudomonas lung infections, macrolide antibiotics can be used as its possess dual properties (Anti-microbial and Anti-inflammatory) and a few side effects.[125]

1.3.6.1 Macrolides

Macrolides are a group of chemical compounds, which have a large lactone ring in their structure (Diagram 9).

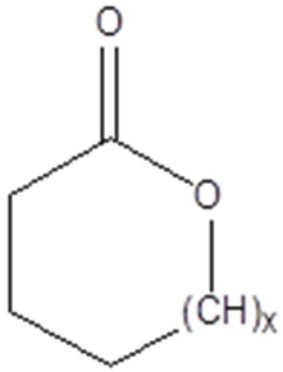


Diagram 9: Lactone Ring Structure

They are mainly classified based on the number of lactone rings: 14-membered compounds (erythromycin and clarithromycin) and 15–membered compounds (azithromycin) (Diagram 10).[126] The 14-membered compound (erythromycin and clarithromycin) consists of lactone ring, oxygen, carbon and monobasic charge.[127] The 15–membered compound (azithromycin) has an azalide ring, oxygen, carbon, nitrogen and dibasic charge. The differences in their structures of macrolides impact their effects on antimicrobial activity and pharmacokinetic profiles.[128] The macrolide antibiotics are widely used in the treatment of

intracellular pathogens, Gram-negative bacteria, and for several Gram-positive bacteria.[129,130] Macrolides play a key role in the treatment of intracellular bacterial infections by marked cell penetration and increased tissue accumulation.[131] They also possess anti-inflammatory properties in both *in vitro* and *in vivo*, thus allowing more clinical benefits.[132]

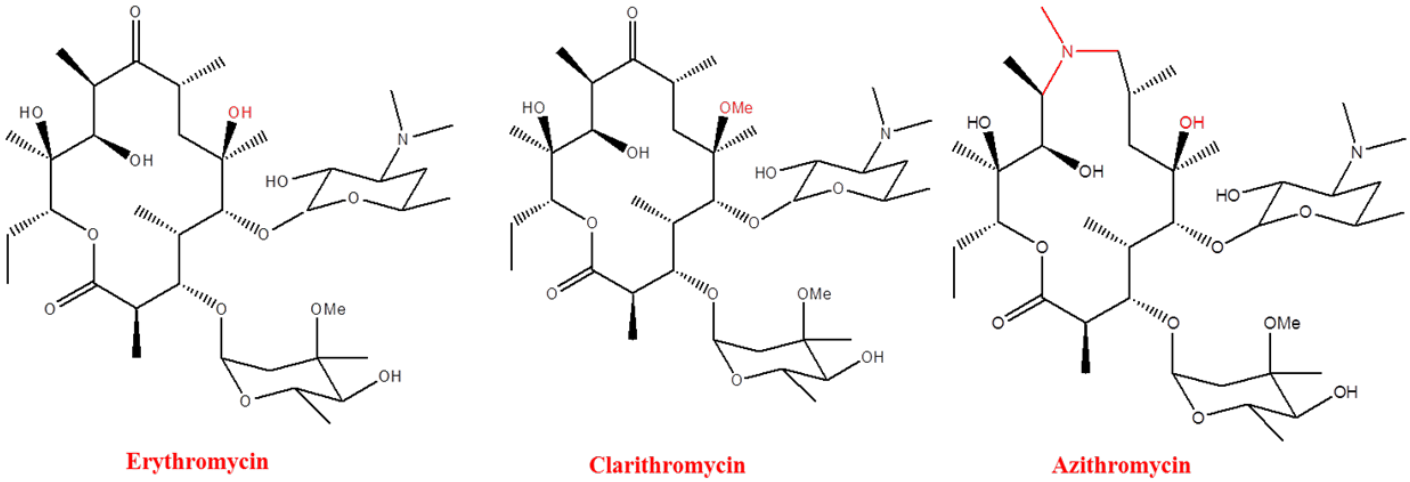


Diagram 10: Chemical Structures of Macrolides

The macrolide antibiotics act as an antimicrobial by reversibly binding to the 50s subunit of the bacterial ribosome, which inhibits the RNA-dependent protein synthesis by preventing the reactions of trans-peptidation and translocation (Diagram 11).[133] The 14 and 15-membered ring chemical structures of macrolides are more likely potent inhibitors of natural mRNA-directed peptide synthesis.[134]

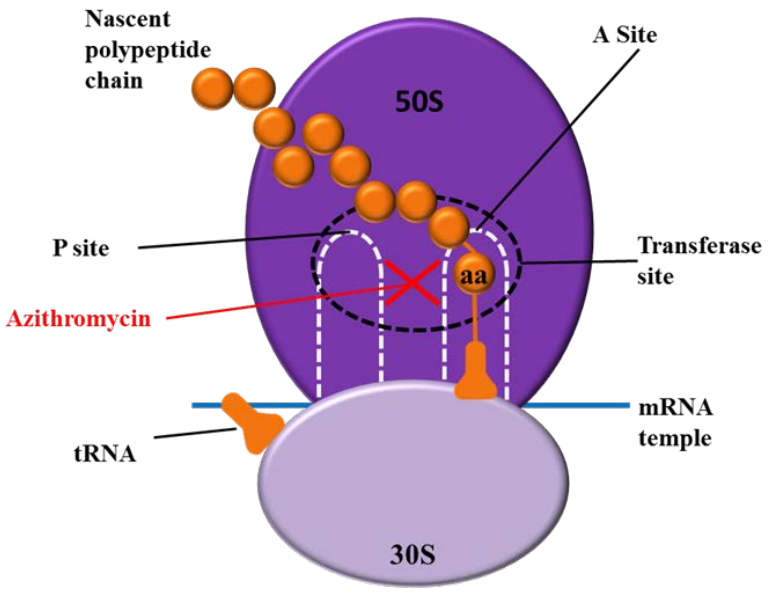


Diagram 11: Mechanism of Action of Macrolides

Erythromycin belongs to the first class of macrolide antibiotics containing 14-membered lactone ring, which adhere to two sugar moieties.[135] Erythromycin is primarily used in the treatment of upper respiratory tract and skin infections.[136] It is also used effectively for the treatment of infections caused by intracellular pathogens like *Legionella, Mycoplasma* and *Chlamydia.*[137] The anti-inflammatory effect of erythromycin also plays an important role in the treatment of lower respiratory tract infections and inflammation, in combination with other drugs.[138]

Clarithromycin is derived from erythromycin by substituting a methoxy group for the C6-hydroxyl group.[127] This substitution in the chemical structure causes the clarithromycin to be more stable in acid, reduces gastrointestinal intolerance and improves bioavailability properties. [139] At sub-inhibitory doses, clarithromycin was effective in the treatment of *P. aeruginosa* biofilms. Compare to erythromycin, it showed similar properties against Gram-negative organisms *in vitro.*[140] Erythromycin and clarithromycin also demonstrated enhanced activity

against *H influenzae* and *S pneumonia.*[141]Moreover, Clarithromycin was used effectively in the treatment of *H pylori*- associated peptic ulcer disease.[142]

Azithromycin has antimicrobial and anti-inflammatory characteristics that makes more effective than erythromycin and clarithromycin.[143] Following the azithromycin pharmacokinetic profile only a single dose regimen per day is require for a short period of time which varies from 3 to 5 days whereas erythromycin and clarithromycin needed two- times dosage regimen for a period of 7 to 14 days.[144] Azithromycin is an second generation, broad-spectrum antibiotic used in the treatment of lower respiratory tract infections.[145] It is developed by inserting methyl-substituted nitrogen in the position of carbonyl group at the 9a position of the aglycone ring.[127] The resulting structure formed after this modification is referred to as an "azalide".[145] This structural modification makes the compound more stable in acid, and increases tissue penetration and serum half-life.[144] This modification also decrease activity against some Gram-positive organisms and increases activity against Gram-negative organisms.[146] The use of azithromycin proved effective anti-mycobacterial effect in the treatment of *Mycobacterium avium* disease in HIV infected patients.[147] Azithromycin can be used as a cell–to-cell blocking agents affecting the formation of biofilm in *P. aeruginosa.*[148] It has been demonstrated that azithromycin at sub-inhibitory concentrations can inhibit *P. aeruginosa* virulence factor levels.[149] Study has shown that azithromycin attenuated alginate production at sub-inhibitory levels in *P. aeruginosa.*[150] In CF infected mice azithomycin exhibited anti-inflammatory effects by reducing cytokine release and cellular infiltration.[151] The study has proven for CF patients that prolonged usage of azithromycin shows more improved clinical outcomes.[152] Azithromycin has the properties of high level accumulation and retention in cells and tissues in comparison to erythromycin.[153,154] Azithromycin accumulates effectively in the phagocytes of cells and increases supply of drug to

the infected sites.[155] The azithromycin dosage of 500 mg/ daily for a 3-day course used for lower respiratory tract infections therapy.[156] A study showed that the typical use of azithromycin is not effective against *P. aeruginosa* infections because of the bacterial outer-membrane impermeability and efflux pump.[157] The usage of azithromycin in higher doses resulted in increased serum levels and hepatotoxicity due to the catalytic ability of cytochrome CYP3A4.[119, 158]

Macrolides exhibit anti-microbial resistance by presence of efflux pumps and target site modification by methylases (Diagram 12). The multidrug efflux pumps of *P. aeruginosa* actively transport antibiotics macrolides out of the cell. MexB efflux pump of *P. aeruginosa* plays a prominent role in anti-microbial resistance of macrolides. The target-site modification by methylation or mutation also prevents the binding of the macrolide antibiotic to its ribosomal target.

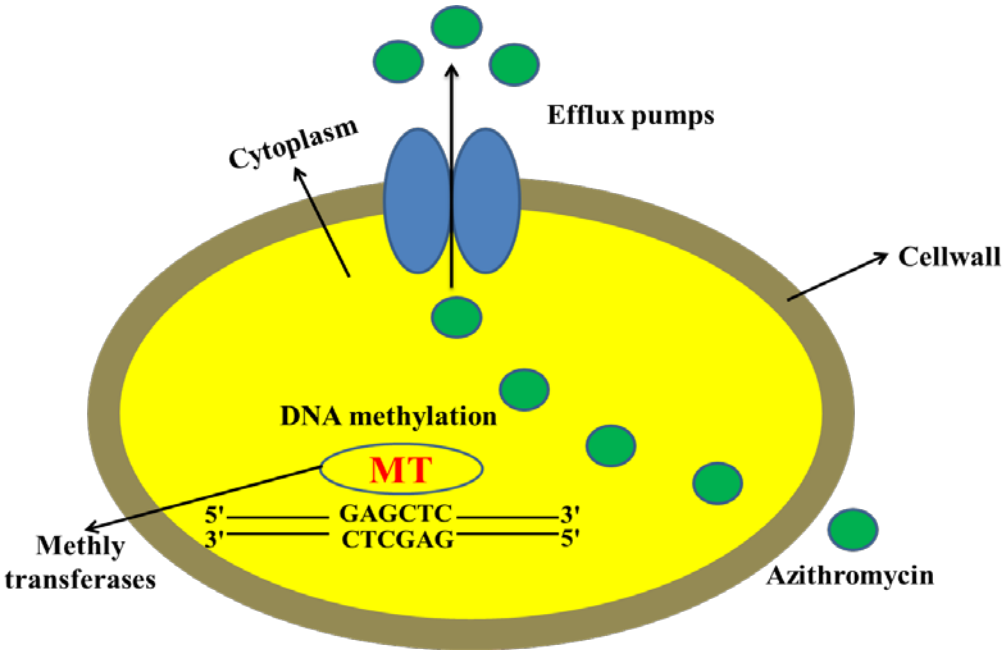


Diagram 12: Resistance Mechanisms of Macrolides

In order to overcome the problem of multi drug resistant *P. aeruginosa* and macrolide toxicities, safe drug delivery systems such as micelles, dendrimers, nanomers, and liposomes are essential.[159] Micelles are self-assembled nanostructures formed in the aqueous solution comprising of amphiphilic copolymer blocks.[160] The major advantages of micelles drug delivery are longer circulation and improved solubility of hydrophobic drugs, although its instability is a drawback. [161]Dendrimers are three dimensional, uniformly distributed macromolecules with hyper branched.[162] Dendrimers are an exemplary drug delivery system due to high water solubility, precise molecular weight, polyvalency and biocompatibility.[163] Solid nano particles are new generation submicron-sized lipid emulsions used alternative to other microparticles.[164] The advantages of solid nanoparticles are increased drug stability, feasibility in loading drugs and easier large scale production, while drawbacks include low drug loading.[165] Among all of the different types of drug delivery systems, liposomes have more leverage because of their

remarkable characteristics such as upsurge therapeutic index of encapsulated drugs and reduces drug toxicity.[166]

1.4 Liposomes

Liposomes are small and round lipid vesicles with sizes ranging from nanometers to micrometers (Diagram 13).[167] Liposome consists of one or more surrounding lipid bilayers and an aqueous core, as well as a hydrophilic head group and hydrophobic tails.[168] Liposomes are considered among the safest and most reliable drug delivery systems since they are biodegradable and biocompatible.[169]

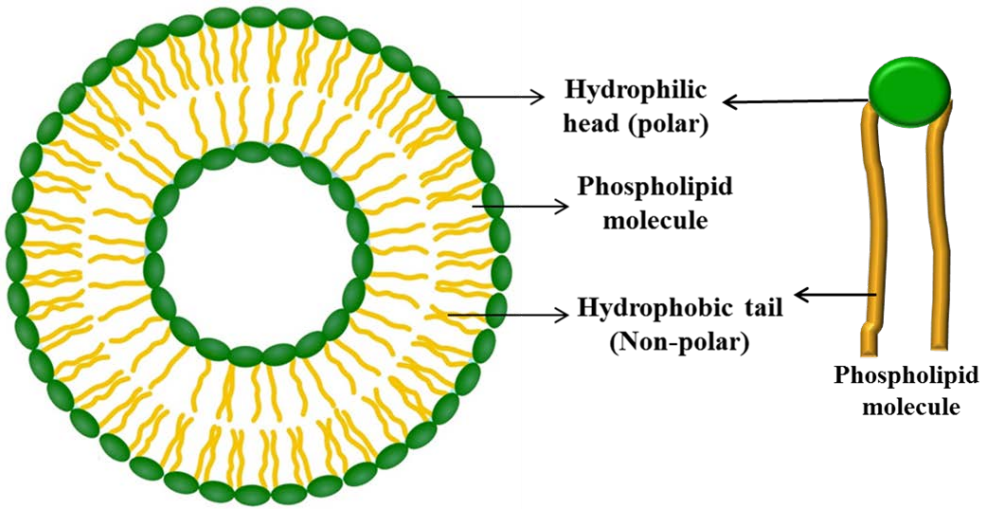


Diagram 13: General Structure of Liposome

Liposomes are broadly classified into three types depending on their size and bilayers: as shown in Diagram 14.

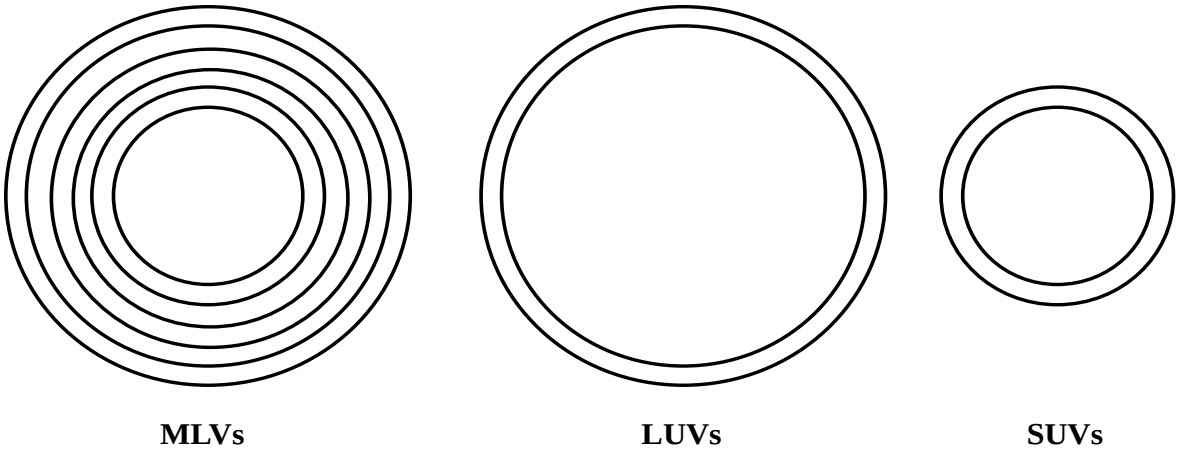


Diagram 14: Classification of Liposome by Size

(a) Multi Lamellar Vesicles (MLVs)

- Usually size ranges about > 0.1 μm
- It consists of more than one bilayer
- Prepared by thin-film hydration method or hydration of lipids

(b) Large Unilamellar Vesicles (LUVs)

- Usually size ranges about > 0.1 μm
- It consists of single bilayer
- Prepared by detergent dialysis method, ether injection method and reverse phase evaporation technique (REV) method

(c) Small Unilamellar Vesicles (SUVs)

- Usually size ranges about ≤ 0.1 μm
- It consists of single bilayer
- Prepared by using sonicator or extruder

1.5 Liposomal drug delivery systems

1.5.1 Phospholipids

Liposomes are composed of natural or synthetic phospholipids. Different kinds of phospholipids are employed in the preparation of lipid vesicles. The most commonly used are phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylserines (PS) (Diagram 15).[170,171] In order to increase the stability and permeability, cholesterol has been used in the bilayer formation of the liposome. Phospholipids have different net charges based on the content of the liposomes such as dipalmitoylphosphatidylcholine (DPPC) which has a neutral charge, dimethyldioctadecyl-ammonium bromide (DDAB) has cationic charges, and dicetyl phosphate (DCP) has anionic charges.[172]

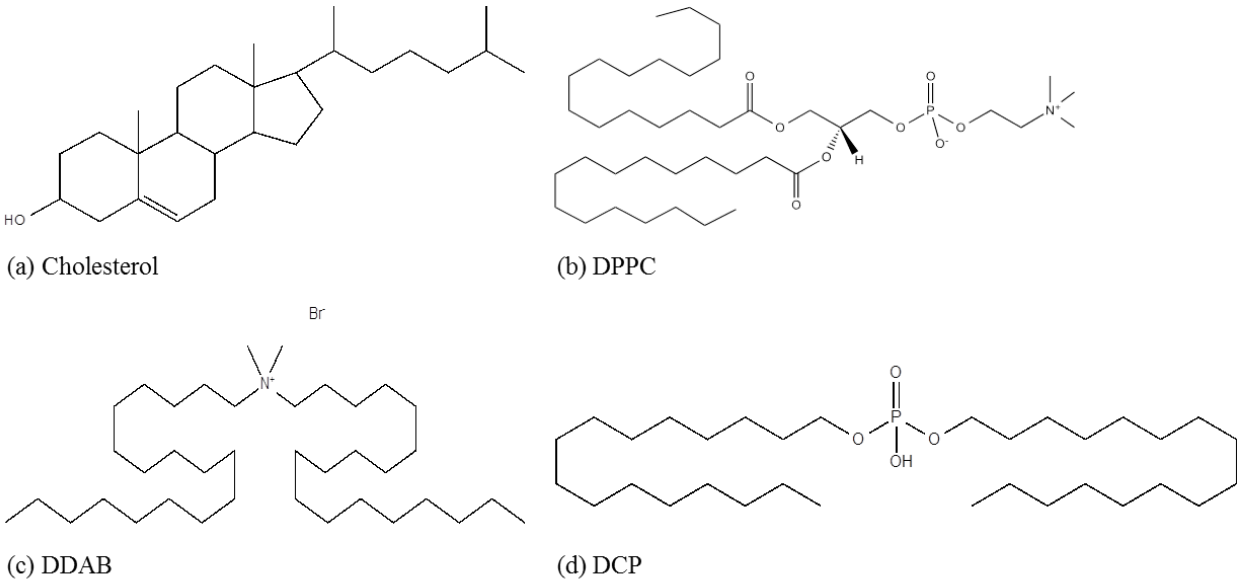


Diagram 15: Structures of Phospholipids

1.5.2 Phase transition temperature

Phase transition temperature is required to change the phospholipid's physical states from ordered gel to disordered gel phase, which occurs due to the conversion of hydrocarbons.[173] Hydrocarbons are tightly packed and fully extended to the disordered stage and make more fluid. The lengths of the hydrocarbons, the charge on the lipids and head group species are the major factors that affect the phase transition temperature of the phospholipids. Increase in the temperature (T_m) of the phospholipid bilayers results in disordered chains making lipids more fluid. The phase transition temperature of the phospholipids can be measured by Differential Scanning Calorimetry (DSC) analysis.[174] Some of the important phase transition temperatures of phospholipids are DPPC (T_m =41° C), DSPC (T_m =55° C) and DMPC (T_m =23°C).[175]

1.5.3 Methods of preparation

Numerous methods have been employed to prepare the liposomes (Diagram 16). The method developed by Bangham is the first and most widely used method in the preparation of multilamellar liposome vesicles (MLVs).[176]

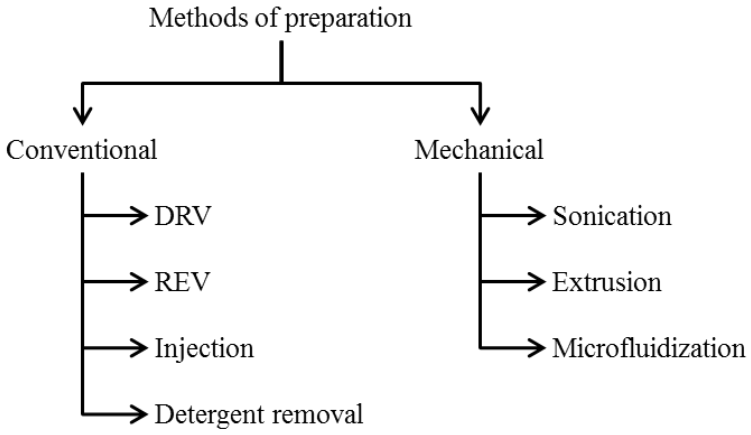


Diagram 16: Methods of Liposome Preparation

1.5.3.1 Conventional methods

Conventional method mainly involves the dissolution of lipids in an organic phase followed by the addition of aqueous solution.[177]

(a) Dehydration Rehydration Vesicles (DRV) method

DRV is the simplest and most widely used method for preparing the liposomes.[178] The initial step in the preparation of DRVs involves dissolving of lipids in organic solvents such as chloroform or a mixture of chloroform/methanol in a round bottom flask. This step is followed by evaporating the organic phase to form a thin layer of lipid film.[179] The following step involves rehydration of the lipid film with an aqueous phase. On rehydration of dried lipid films the lamellae are formed, the last step involves applying mechanical agitation such as shaking or vortexing to detach the lipid film from the flask. The key advantage of this method is simple, higher encapsulation efficiency and more stability. Different varieties of lipids and drugs can be encapsulated using this method. I used DRV method for the preparation of liposomes in this study as it provides higher encapsulation efficiency of loading macrolide drug azithromycin. The major drawbacks of this method are lower entrapment of drugs and retention.

(b) Reverse Phase Evaporation (REV) method

The preparation of liposomes by REV method involves high aqueous space, which enables to entrap a high amount of the aqueous material.[166] The liposomes prepared through the REV method can be made from different varieties of lipids or lipid mixtures. The liposome preparation by this method involves two phases; First water-in-oil emulsion is formed by the limited sonication of a two- phase system containing phospholipids in different organic solvents

such as diethyl ether or isopropyl ether. Later, the organic solvents are removed by reduced pressure generating viscous gel. Under reduced pressure conditions and with continuous rotary evaporation the excess formed solvent is removed resulting in the formation of liposomes.[180] The major advantages of this method are effective loading of macromolecules, while the drawbacks are a need for organic chemicals, and mixtures of LUVs and MLVs are formed.

(c) Injection method

The preparation of liposomes by injection method is done though ethanol/ether.[181] The lipids are dissolved in the ethanol or ether and the lipid solution is slowly injected in to the aqueous phase. The next step involves the evaporation of organic solvent, resulting in the formation of unilamellar liposomes. The major advantages of this method include sterility, can be done heating and narrow vesicles of liposome can be obtained.[182]

(d) Detergent removal method

In this method of liposome preparation, different kinds of detergents are used in lipid solubility.[183] The detergent is removed by methods such as dialysis, column chromatography and adsorption, resulting in the formation of micelles, which then leads to formation of liposomes. The significant advantage of this method is its extreme flexibility, which allows for the preparation of various varieties of liposomes.

1.5.3.2 Mechanical methods

In these methods, mechanical force is applied in order to alter the liposome size and lamellarity.[184]

(a) Sonication

It is the first most widely used and least complicated method in the liposome size reduction process by mechanical treatment. The two major techniques used in the preparation are: i) probe sonication; and ii) bath sonication. Probe sonicator is a better method to prepare SUVs on a small scale, because the process involves high energy input into lipid dispersions, which can be applied directly on the MLVs.[166] Bath sonicator is a commonly method used in the preparation of liposomes for large quantity scale. It is the most widely used method for the small-scale preparation of SUVs with the highest input of energy into lipids.

(b) Extrusion

The extrusion method of preparing liposomes involves liposome size reduction by passing them through the polycarbonate filters of different pore sizes with repeated cycles of extrusion under moderate pressure.[185] Extrusion is a simple technique without use of any volatile organic compounds, but requires high-pressure nitrogen gas in the extruder. Drawbacks include a more time consuming process of size reduction.

(c) Microfluidization method

Microfluidizer/Microemulsification is a technique widely used in the large scale production of liposomes in the pharmaceutical industry.[186] The microfluidizer pumps fluid at high pressure along channels of small size, which later divides the fluid into two streams, resulting in collision at high velocity. Factors like turbulence and cavitation result in the production of small size liposomes and polydispersity. The disadvantage of this method is requirement of high pressure for size reduction causing lipid degradation.

1.5.4 Methods of characterization

(a) Particle size

The particle size and distribution (polydispersity index) of the liposomes are important parameters for characterizations. Particle size affects the therapeutic use and mode of administration. The size of the liposome is also related to the RES uptake. Dynamic Light Scattering (DLS) is one of the suitable methods to measure the particle size.[187] The principle involved in DLS method is diffusion co-efficient based on Brownian motion.[188] Key drawbacks of this DLS technique are that it can measure only the particle size but does not give any information regarding the morphology of particles, as well as its limitations in measuring the polydispersity samples. The electron microscopy is an alternative method used to characterize the particle size. It is an enhanced technique, however, and some limitations with the sample size. It have is not suitable for monitoring the stability of the sample. The size of liposomes can be reduced through methods like sonication, extrusion, and micro fluidization techniques.[189]

(b) Lamellarity

Lamellarity is a parameter used to measure the average particle size in relation to the amount of drug entrapped inside the liposome.[190] Lamellarity is mainly done by phosphorus nuclear magnetic resonance (P-NMR) technique, which is mainly based on the signals response to the addition of magnesium ions (Mn^{2+}). Other methods such as NMR, Small Angle X-ray Scattering (SAXS) and Electron microscopy are used to characterize the zeta potential of liposomes.[191]

(c) Zeta potential

The zeta potential is an important parameter used in the characterization of liposomes to measure the overall charge of the liposomes.[166] Zeta potential has an effect on pharmacokinetic properties of liposomes in the body and also impact the phagocytosis of the liposomes in the blood stream.[192] It is also used to measure the stability of the liposomes and helps in understanding the controlling factors like fusion and precipitation of liposomes.[166]

(d) Encapsulation efficiency

Encapsulation efficiency is an important method used to measure the quantity of drug entrapped inside the liposomes that is commonly used to optimize the formulation.[193] Encapsulation efficiency is generally defined as a percentage of entrapped drug to the initial concentration of drug in the solution. The lipophilic drugs are usually entrapped in the lipid bilayers and hydrophilic drugs are incorporated in the aqueous core. Triton X 100 is a detergent used to rupture the vesicles during the measurement of encapsulation efficiency.[193] Alternate methods like High Performance Liquid Chromatography (HPLC), spectrophotometry and microbiological assays are also used to determine the encapsulation efficiency.

1.5.5 Applications of liposomes

Liposomes are used as a drug delivery system due to unique and special characteristics.[194,195] The liposomal drug delivery system differs from other drug carriers as it releases the drug either in the blood plasma or at the site of application.[196] The liposomal drug delivery systems for pulmonary delivery have been widely used in the treatment of CF by encapsulating different kinds of drugs such as antimicrobials, anti-oxidants, and cytotoxic.[197]

1.5.5.1 Liposomal formulations in the market or in clinical trial pipeline

In recent years, there has been a tremendous development in liposomal technology. Many of these liposomal formulations are under clinical trials, and a few have already approved for human use by the US FDA (Table 1).[198, 199] Purpose of using liposomal drug delivery systems is to reach the target selection of active drug in different sites of the carcinoma and inflammated areas.[200,158] In cancer treatment, liposomal products such as Doxil® and DaunoXome® have been approved successfully at first for the treatment of Kaposi Sarcoma.[201,202] For serious fungal infections, the liposomal formulations such as Ambisome®, Amphacil® and Abelcet® have also been approved.[203] AmBisome® was approved for the treatment of aspergillosis, cryptococcosis and candidiasis.[204] Recently, the FDA approved vincristine liposomal formulation Marqibo® for the treatment of acute lymphoblastic leukemia.[205]

Table 1: Approved liposomal products for human usage

Product	Drug	Lipid Composition	Route of Administration	Manufacturer	Approved treatment	References
AmBisome	Amphotericin B	HSPC,DSPG & CHOL	Intravenous	Gilead Sciences (USA)	Fungal infections	[206, 204]
Doxil	Doxorubicin	HSPC, CHOL & DSPE-PEG_{2000}	Intravenous	Johnson & Johnson (USA)	Kaposi's Sarcoma, Breast and Ovarian cancer	[207, 208]
Lipo-Dox	Doxorubicin	HSPC, CHOL & DSPE-PEG_{2000}	Intravenous	Sun Pharma (India)	Kaposi's Sarcoma, Breast and Ovarian cancer	[209, 210]
Myocet	Doxorubicin	EPC & CHOL	Intravenous	Sopherion Therapeutics (USA)	Breast cancer	[211, 212]
DepoCyt	Cytarabine	DOPC, DPPG, CHOL & Triolein	Spinal	Pacira Pharma (USA)	Lymphomatous meningitis and neoplastic	[213, 214]
DepoDur	Morphine	DOPC, DPPG, CHOL & Triolein	Epidural	APP Pharma (USA)	Pain treatment	[215, 216]
DaunoXome	Daunorubicin	DSPC & CHOL	Intravenous	Galen (USA)	Kaposi's Sarcoma, Blood cancer	[217, 218]
Marqibo	Vincristine	Egg sphingomyelin & CHOL	Intravenous	Talon Therapeutics (USA)	Acute lymphoblastic leukaemia	[219, 220]
Visudyne	Verteporphin	EPG & DMPC	Intravenous	Bausch & Lomb (USA)	Photodynamic therapy	[221, 222]

In the pipeline, two liposomal formulations for the treatment of *P. aeruginosa* infections are shown in (Table 2), Liposomal amikacin formulation (Arikace) in phase III clinical trial is used by inhalation for the treatment of CF, Non CF and bronchiectasis.[223] Another formulation in phase III clinical trial, Liposomal ciprofloxacin (Lipoquin) manufactured by Aradigm and administered through inhalation is intended for the treatment of CF, Non CF, and bronchiectasis.[224,225] The clinical results of these formulations for treating *P. aeruginosa* infections in CF patients provided positive results for further investigations.

Table 2: List of liposomal products in clinical trials stage for the treatment of *P. aeruginosa* infections

Product	Drug	Lipid mixture	Route of Administration	Manufacturer	Target treatment	Stage	References
Arikace	Amikacin	DPPC & CHOL	Inhalation	Insmed (USA)	Lung infections	Phase III	226, 227
Lipoquin	Cipro floxacin	HSPC & CHOL	Inhalation	Aradigm (USA)	Lung infections	Phase III	228, 229
Pulmaquin	Cipro floxacin	HSPC & CHOL	Inhalation	Aradigm (USA)	Lung infections	Phase III	230, 231

1.5.5.2 Applications of Liposomal Antibiotics—Evidences from *In vitro* and *In vivo* studies:

The key focus of developing new liposomal antibiotic formulations is to increase the therapeutic efficacy of drugs at the site of infection and reduce toxicity.[231] Liposomes are the most widely used nanodevices for the encapsulation of some antibiotics for the treatment of several intracellular and extracellular infections (Table 3).[232] Research suggest that liposomal encapsulated antibiotics have less toxic effect than the conventional administration of

antibiotics.[233] Less frequency of dosing and increased fusion of antibiotic with infected cells are major significant characteristics of the liposome-encapsulated antibiotics.[234]

Table 3: Liposomal antibiotics used for various bacterial infections

Liposomal entrapped antibiotics	**Bacteria**		**References**
	In vitro	***In vivo***	
Azithromycin	*P. aeruginosa*, MAC		*158, 235*
Clarithromycin	*P. aeruginosa*, MAC	MAC	*236, 237, 238, 239*
Erythromycin	*P. aeruginosa*		*193, 240*
Amikacin	*P. aeruginosa, B. cenocepacia,* MAC, *M. tuberculosis, S. aureus*	MAC, *M. tuberculosis*	*241, 242, 243, 244,245, 238*
Gentamicin	*P. aeruginosa, B. cenocepacia,* MAC, *E. coli, K. pneumoniae*	*K. pneumoniae, S. enterica, B. abortus*	*246, 247, 248 249, 250*
Tobramycin	*P. aeruginosa, B. cenocepacia, B. cepacia, S. aureus E. coli, S. maltophilia*	*P. aeruginosa, B. cepacia*	*251, 252, 241 242, 253, 254*
Vancomycin	*S. aureus, S. epidermidis,* MRSA	MRSA	*255, 256, 257*
Polymyxin B	*P. aeruginosa, E. coli, K. pneumoniae, B. bronchiseptica*	*P. aeruginosa*	258, 259, 260 261

Enormous research has been done for the application of liposomes as drug delivery systems by *In vitro* and *In vivo* studies in the treatment of several infections. The liposomes show increased drug concentrations at the site of infection along with reduced toxic effects.[262] One study has shown that erythromycin was able to be encapsulated in liposomes with higher efficacy and more stability.[193] The encapsulation of liposomes with antibiotics like aminoglycosides and macrolides can enhance their effect in the treatment of *P. aeruginosa* infections.[248] Compared to free tobramycin, liposome- loaded tobramycin shown effective in eradicating Gram-negative bacteria.[263] Furthermore, clarithromycin loaded liposome demonstrated increased antibacterial effects against *P. aeruginosa* and decreased toxic effects on the human lung cells.[236] For *M. avium* infections, liposome encapsulated azithromycin or ciprofloxacin focus on the reticuloendothelial system, which provided efficient direct delivery of antibiotics into the infected cells resulting in a more effective treatment.[235] The liposomes loaded with aminoglycoside antibiotics showed admirable results in the treatment of bacterial infections. Liposomal Amikacin for Inhalation (LAI) is a new formulation under clinical trial showing promising results to maintaining lung function and reduced pulmonary exacerbations.[264] The encapsulation of amikacin in liposomes resulted in a higher concentration of the drug in infected tissues in murine model of *M. avium* infected mice.[265]

The major drawbacks of liposomal antibiotics are lower encapsulation efficiency, shorter shelf-life and sterility problems.[231] The encapsulation efficiency is a considerable factor in the therapeutic efficacy of liposomes.[266] The preparation of liposomes by DRV method demonstrated higher encapsulation efficiency with a better stability of macrolides and aminoglycosides.[193] Shorter shelf-life of liposomes can be influenced either by physical or chemical processes. Few studies reported that upon intravenous administration of liposomes it

could lead to lower stability and short circulation in the blood stream.[267] The sterilization process of the phospholipids used in the preparation of liposomes is a time consuming and expensive method of liposome preparation at a larger scale. These are some of the major disadvantages of liposomal drug delivery systems, and they have not yet been addressed scientifically.[166,262]

For future prospects, developing new liposomal antibiotic formulations is essential for the treatment of CF caused by *P. aeruginosa* infections. The unique characteristics of liposome as a drug delivery system advance the development of new liposomal formulations with more biocompatible and therapeutic efficacy of drugs.

1.6 Thesis objective

The objectives of this research study are:

- To characterize liposome encapsulated azithromycin formulation and evaluate its antimicrobial effects against *P. aeruginosa.*
- Examine the liposomal azithromycin effects on biofilm activity and virulence factors, quorum sensing reduction, motility at sub-inhibitory levels.
- To investigate the mechanism of bacteria and liposome interactions.

Chapter 2

2 Antimicrobial Properties of Liposomal Azithromycin for *Pseudomonas* Infections in Cystic Fibrosis Patients

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Abstract

Objectives: This work has been carried out to construct a novel liposomal azithromycin formulation and examine its antimicrobial effects against *P. aeruginosa.*

Methods: The liposomal azithromycin formulation was prepared by the dehydration-rehydration vesicle method and its characterisations were tested. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of liposomal formulation was determined by microbroth dilution method. Liposomal azithromycin activity against biofilm forming *P. aeruginosa* was assessed using Calgary biofilm device. The effect of subinhibitory concentrations of liposomal azithromycin on bacterial virulence factors and motility studies was performed on *P. aeruginosa* strains. The bacteria and liposome interactions were studied by using flow cytometry analysis. The toxicities of the liposomal formulation on erythrocytes and A549 lung cells were evaluated *in vitro.*

Results: The average diameter of the liposomal azithromycin was found to be 406.07$\pm$ 45 nm and encapsulation efficiency was 23.8 ± 0.2 %. The MIC and MBC values of liposomal azithromycin were significantly lower than the free azithromycin. The liposomal azithromycin significantly reduced the bacteria in the biofilm and attenuated different virulence factors production; it also reduced the different patterns of bacterial motilities. By flow cytometry analysis data, it was shown that there are interactions of liposomes with the bacterial membranes. No significant hemolysis or cell toxicity was observed with liposomal formulation.

Conclusions: The results of this research indicate that this novel liposomal azithromycin formulation could be a useful therapy to enhance the safety and efficacy of azithromycin against *P. aeruginosa* infected persons.

Keywords: Cystic fibrosis, Liposomes, Azithromycin

2.1 Introduction

Cystic fibrosis (CF) is a life-threatening autosomal recessive hereditary disorder, which usually affects Caucasian populations in the ratio of one among 2,000 newborns.[268-270] It is also considered as a multi-functional or multi-systemic disease, as it affects several organs and systems of the body like liver, pancreas, gastrointestinal tract, reproductive system and lungs in particular.[3,271] Among all these organs, pulmonary injury is mainly responsible for patient death in the cystic fibrosis population.[4] Recurrent pulmonary infection and inflammation are the major risk factors associated with cystic fibrosis disease.[272] Cystic fibrosis is caused by the mutations in the cystic fibrosis transmembrane conductance (CFTR) gene.[273] Approximately 2000 CFTR mutations have been identified that can be classified into 6 types based on their effect.[13] The deletion of phenylalanine in the amino acid position at Δ508 is the most common mutation, which is responsible for the majority of cystic fibrosis cases.[14] The normal function of CFTR in lungs is to regulate the water and salts across the epithelial cells, whereas in the abnormal or dysfunctional CFTR in the lungs there is hyper absorption of sodium from the epithelial surface, which results in the depletion of airway surface liquid on the surface.[274] All these changes in airway surface depletion lead to the prevention of normal ciliary beating and accumulation of more thick and sticky mucus on the airway surface, which leads to favorable conditions of microbial colonization. [37] The major opportunistic pathogens implicated in pulmonary infection

are Gram-positive *Staphylococci* species and Gram-negative *Pseudomonas aeruginosa* and *Burkholderia cenocepacia.*[31] The Toxic Shock Syndrome Toxin-1 (TSST-1), coagulases and proteases are the virulent factors produced by the *Staphylococci* species.[275] The *P. aeruginosa* releases large number of virulence factors like lipase, chitinase, elastase, protease, exotoxin A, neuraminidase, catalase and superoxide dismutase.[276,277] Siderophores, lipases and proteases are the virulence factors released by the *B. cepacia.*[278]

Among all bacteria, the most opportunistic human pathogen is *P. aeruginosa* mainly responsible for pulmonary exacerbation in CF population.[279,280] It is a Gram negative bacillus, which mainly affects the lower respiratory tract of the lungs; a ubiquitous organism mainly found on the surface of plants, water, soil and hospital areas.[281] It is the most prevalent respiratory bacterial pathogen that affects cystic fibrosis individuals with compromised immune systems.[282] Nowadays it is called 'superbug' because of the *P. aeruginosa* engendered resistance.[283] *P. aeruginosa* exhibits different kinds of resistance mechanisms by outer membrane impermeability, and through efflux of the antibiotic.[134,284] *P. aeruginosa* comprises a mechanism called quorum sensing (QS) which is cell-cell communication of signaling molecules produced in response to bacterial density.[69] Quorum sensing signaling molecules regulates gene expression that controls the formation of biofilm and production of different virulence factors thereby contributing to *P. aeruginosa's* tolerance to anti-microbial chemotherapy.[74,75] Virulence factors like lipase, chitinase, elastase and proteases released from *P. aeruginosa* can cause tissue damage, proteolysis imbalance and are thought to be leading cause of immunomodulation.[77,285]

Biofilm formation is one of the major systems by which bacteria bypass the chemotherapy.[286] Biofilms are a group of organisms that grow attached to a surface and are embedded in an extracellular matrix composed of proteins and exopolysaccharides.[74] *P. aeruginosa* biofilms can

cause chronic infections due to increased tolerance to antibiotics and resistance to phagocytosis, as well as components of the immune system including innate as well as adaptive resulting in immune complex mediated chronic inflammation.[286]

Antibiotics such as macrolides are widely used in the treatment of pseudomonal lung infections.[33,178,287] Macrolides are a group of compounds, which have a large lactone ring in their structure.[134] The major drug of choice among macrolides is azithromycin, a second generation, broad spectrum antibiotic usually used in the treatment of lower respiratory tract infections.[288,289] Azithromycin accumulates effectively in the phagocytes of cells and increases supply to the infection sites.[131] Azithromycin acts by binding to the 50S subunit of the bacterial ribosome, thus inhibiting bacterial protein synthesis. Azithromycin can be used as cell–to-cell blocking agents affecting the formation of biofilm in *P. aeruginosa*.[290] In lung diseases such as cystic fibrosis it has been proven that azithromycin shows more improved clinical outcomes in patients with extended usage.[119] On the contrary, study demonstrated that the typical use of azithromycin is not effective against *P. aeruginosa* infections because of the bacterial outer-membrane impermeability and efflux pump.[33] The main mechanism of macrolide resistance in *P. aeruginosa* biofilms is due to hyperexpression of MexCD-OprJ efflux pump, which plays an important role in the antibiotic resistance.[291] The MexCD-OprJ efflux pump is mainly expressed due to mutations in gene NfxB, which result in the increase of efflux genes and encodes a repressor of MexCD-OprJ efflux pump expression.[93,292,293] The higher doses of azithromycin usage also resulted in increased serum levels and hepatotoxicity due to the failure catalytic ability of cytochrome CYP3A4.[159] It has been reported that 40 different hydrolytic enzymes including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, sulfatases and many proteins of yet unknown function have been identified to made up of lysosomal

content,[294] among these phospholipase A_1 is majorly responsible for the drug induced phospholipidosis by azithromycin.[295] In addition, the accumulation of azithromycin in lysosomes may hinder with phospholipase A_1 activity, resulting in storage disorder phospholipidosis indicating excess accumulation of phospholipids in the tissues causing inflammatory reactions and histopathological alterations in the organs.[119,296-298]

To overcome these problems of *P. aeruginosa* resistance to azithromycin, toxic effects and its atypical pharmacokinetics,[159] new drug delivery systems like liposomes have been developed. Liposomes are considered to be safe, reliable and biodegradable drug delivery systems as they are mainly made up of phospholipids and its derivatives, which could increase the efficacy and reduce the toxicity associated with the drugs.[166,299,300] They are small and round lipid vesicles with sizes varying from nanometer to micrometer range.[301] It consists of one or more lipid bilayers surrounded by an aqueous core.[302] It also has a hydrophilic head and hydrophobic tail along with the lipid bilayers.[303] Usually hydrophilic drugs are incorporated into the aqueous core and lipophilic drugs into the bilayers of the phospholipid.[304,305] The liposomes show increased drug concentrations at the site of infection along with reduced toxic effects.[166,201] Previous work done by our group showed that erythromycin was able to be encapsulated with higher efficacy and more stability.[193] Furthermore, Alhajlan *et al* showed that clarithromycin loaded liposome exhibited an increase of antibacterial effects against *P. aeruginosa* and a decreased toxic effect on the human lung cells.[236] Oh *et al* revealed that liposome encapsulated azithromycin or ciprofloxacin focus on the reticuloendothelial system, which in turn provide efficient direct delivery of antibiotics into the infected cells resulting in a more effective treatment for *M. avium* infections.[235]

In the current work we developed, characterised and evaluated a novel liposomal azithromycin formulation for its antimicrobial effects against *P. aeruginosa*. Liposomal azithromycin activity against biofilm forming *P. aeruginosa* was assessed and the effect of subinhibitory concentration of free and liposomal azithromycin was studied on *P. aeruginosa* quorum sensing, virulence factors and bacterial motility. In addition, the liposome and bacterial interactions was investigated. Furthermore, toxicities of the liposomal formulation on erythrocytes and cultured A549 lung cells were evaluated *in vitro.*

2.2 Materials and methods

2.2.1 Chemicals and media

Azithromycin was obtained from Bonn Schtering Bio Sciences (Pondicherry, India). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, Triton X-100, PKH2-GL kit, trypan blue, elastin-congo red, chitin azure, β-D-galactopyranoside, RPMI (Roswell park memorial institute medium), DPBS (Dulbecco's phosphate buffered saline) and agarose, were purchased from Sigma-Aldrich (Oakville, ON, Canada). Tryptic soy agar, tryptic soy broth, Luria-Bertani (LB) broth, and Luria-Bertani agar were obtained from Becton Dickinson Microbiology Systems (Oakville, ON, Canada). Cell viability assay kit was purchased from Fisher Scientific (Ottawa, ON, Canada). Cationic-adjusted Mueller-Hinton (CAMH) broth for culturing microorganisms was purchased from Beckton Dickinson (Franklin Lakes, NJ).

2.2.2 Cell culture

A549 human lung carcinoma epithelial cells were obtained from the American Type Culture collection (ATCC CCL-185, Manassas, USA) and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS); this toxicity study was done without the addition of antibiotics. The cells were grown to 85% confluence in 5% CO_2 at 37°C and maintained using traditional cell culture techniques.[306]

2.2.3 Bacterial strains

Bacillus subtilis (ATCC 6633) laboratory strain was used as an indicator for testing of azithromycin activity. *Pseudomonas aeruginosa* strain (PAO1) was generously donated by Dr. R.E.W. Hancock (University of British Columbia) and Clinical isolates of *P. aeruginosa* (PA-1, PA-5, PA-3, PA-7, PA-M13640, PA-M13641-1, PA-M13641-2, PA-48912-1,PA-48912-2) were purchased from PML Microbiologicals (Mississauga, ON, Canada) and some were obtained from the Memorial Hospital's Clinical Microbiology Laboratory (Sudbury, ON, Canada). All the bacterial strains were stored at -80°C in cationic-adjusted Mueller-Hinton (CAMH) broth supplemented with 10% glycerol. All strains were grown for 18 h in CAMH broth prior to the minimum inhibitory concentrations **(**MIC) and minimum bactericidal concentrations (MBC), QS, and virulence factors experiments. For the Acyl homoserine lactones (AHL) detection *Agrobacterium tumefaciens* strain A136 (pCF218)(pCF372) (Ti-) was used as the biosensor and cultured in Luria-Bertani (LB) broth at 30°C.

2.2.4 Preparation of liposomes

The dehydration-rehydration vesicle (DRV) method was used for the preparation of liposomes. 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol in a molar ratio of 6:1 (lipid to cholesterol) was used. DPPC (0.11382 g) and cholesterol (0.01 g) were added to a round bottom flask and then dissolved in sufficient amount of chloroform: methanol (2:1) mixture. The mixture in the round bottomed flask was dried to a lipid film with a rotary evaporator (Rotavapor; BÜCHI Labortechnik AG) in a water bath at 41°C under controlled vacuum (V-800, Brinkman). 0.03 g of azithromycin dissolved in phosphate buffered saline (PBS, pH 7.4) was added to rehydrate the lipid film. The lipid suspensions in PBS were vortexed for five minutes and then sonicated for 2 minutes (cycles of 45 sec ON and 10 sec OFF) in an ultra-sonic dismembrator bath (FS20H; Fisher Scientific, Ottawa, Canada) with amplitude of 45Hz (Model 500, Fisher Scientific). The lipid suspension was divided into aliquots of tubes. The tubes were frozen for 15 min then freeze dried in freeze dry system (Model 77540, Labanco Corporation, Kansas city, MO, USA). The obtained powdered formulations were stored in a freezer at 0°C until use. For rehydration, 100 µL of PBS was added and the mixture was vortexed and incubated for 5 min at 40°C. This step was repeated thrice and finally 700 µL of PBS was added to make it 1000 µL of suspension. The excess of unencapsulated drug was removed following three rounds of washing with PBS using centrifuge (16000 x *g* for 15 min at 4°C).

2.2.5 Microbiological assay for the measurement of azithromycin

To measure the encapsulation efficiency (EE) of azithromycin into liposomal vesicles laboratory strain *Bacillus subtilis* (ATCC 6633) was used as an indicator organism as suggested

by the Clinical and Laboratory Standards Institute (CLSI). Agar diffusion assay was used to quantify the concentrations of azithromycin incorporated into liposomes.[306] *Bacillus subtilis* (ATCC 6633) was cultured overnight in CAMH broth, and a bacterial solution was prepared equivalent to 0.5 McFarland standards (1.5×10^8 cfu/mL) . The bacterial cells were then added to an autoclaved molten agar solution at 41°C and immediately discharged into a sterile glass plate (440 mm × 340 mm) to form a thin layer of agar and bacteria. The liposomal azithromycin sample was centrifuged at 12,000 × *g* for 20 min at 4°C and Triton X-100 in PBS 0.2% (vol/vol) was added to the obtained pellet in order to release the drug from the liposome.[307] We must point out that this concentration of Triton X-100 (0.2%) has no effect on the bacterial growth. A well-puncher device is used to make wells of 5 mm in diameter and filled with 25 µL of liposome samples or standard solutions of azithromycin, and the glass plate was incubated for 18 h at 37°C.[308,309]After the incubation period of 18 h, inhibition zones obtained in the plate were measured in triplicates. In order to quantify the encapsulation efficiency of the liposomal azithromycin formulations, average values of the triplicates were used. The sensitivity of the microbiological assay was found to be 0.00390 mg/L and quantifiable limit for azithromycin was 0.003 mg/L. For azithromycin, the standard curve linearity extended over the range of 0.003 to 2 mg/L and gave a correlation coefficient greater than 0.99. The concentrations of the obtained measurements are the means of at least three independent experiments measured in triplicate for each experiment.

2.2.6 Determination of encapsulation efficiency

The encapsulation efficiency of liposomal azithromycin was determined as the percentage of azithromycin entrapped in the liposomes (determined by the microbiological assay

as mentioned in the previous paragraph) respective to the initial amount of the azithromycin in solution.[248] The encapsulation efficiency is measured by the formula:

Encapsulation efficiency (%) $= C_{DRVs}/C_{sol}$

C_{DRVs} = Concentration of the antibiotic entrapped in dehydration-rehydration vesicles

C_{sol} = Initial concentration of the antibiotic added in to the mixture

The concentration of the entrapped azithromycin in the liposome was determined by the microbiological assay as mentioned above.

2.2.7 Determination of particle size and polydispersity index (PI)

The mean diameter of liposomes and the polydispersity index (PI) were measured by using a Submicron Particle Sizer Model 270 (Nicomp, Santa Barbara, CA).[173,308]The liposomal azithromycin samples obtained after rehydration by adding 1000 µL of PBS were subjected to particle size analysis. Liposomal azithromycin were diluted in clear glass tubes with double distilled water to get sufficient reading between ranges of 250 to 350 kHz for the photo pulse to obtain the particle size data. The process was repeated 2-3 times with the liposomal azithromycin sample to take average value of the particle size data.

2.2.8 Differential scanning calorimetry (DSC) Characterisation

DSC analysis was performed using the TA Instruments Q100 Differential Scanning Calorimeter (Inglehart road, Grimsby, Ontario, Canada). A scan rate of $10°C \cdot min^{-1}$ was employed with a temperature range of 25℃ -200℃. Approximately 5-7 mg sample was used for analysis; using an empty pan as reference. Pure DPPC, Physical mixture-1 (DPPC, Cholesterol),

Physical mixture-2 (DPPC, Cholesterol and Azithromycin) and azithromycin loaded liposome samples were prepared for thermal analysis.[310] The measurements of each sample were repeated three times. The main phase transition temperatures were determined using TA universal analysis 2000 program.

2.2.9 Stability studies of liposomal azithromycin

The stability study of liposomal azithromycin was evaluated in PBS at 4℃ and 37℃. The stability of liposomal azithromycin was measured as the percentage of retention of the initial encapsulated azithromycin after the incubation period of time at 4°C and 37°C.[248] Briefly, liposomal azithromycin was suspended in PBS and incubated in water bath shaker with mild agitation at 100 rpm (Julabo SW22 Incubator Shaker; Labortechnik, Seelbach, Germany). After incubation times of 0, 2, 4, 6, 8, 24, and 48 h, samples were centrifuged at 18,000 × *g* for 15 min at 4°C to remove the the released drug.[248,252]The supernatants of the liposomal azithromycin samples were collected, and 25 µL was transferred in to puncher-made holes on a plate containing agar prepared with *B. subtilis* ATCC 6633. The plates were then incubated at 37°C for 18 h, and the inhibition zones were measured.

2.2.10 Determination of the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

The MIC was done on clinical strains of mucoid and non-mucoid *P. aeruginosa* (PA-1, PA-5, PA-3, PA-7, PA-M13640, PA-M13641-1, PA-M13641-2, PA-48912-1, PA-48912-2) by the microbroth dilution technique. The bacterial strains were subjected to 0.5 McFarland standards and transferred to 96 well plates as reported previously.[306] The bacterial strains were exposed to different dilutions of azithromycin in the plate. The plates were incubated for 24 h at

37°C.[44] After incubation MIC results were noted by visually observing the point at which a particular concentration of drug inhibits the growth of organisms. The selected concentrations were subjected to MBC method by inoculum the sample to the agar plates and incubated for 24 h at 37°C to evaluate the growth of bacteria on the plates.[247] The results were taken from three separate experiments.

2.2.11 Effect of subinhibitory concentrations of free and liposomal azithromycin on the growth of *P. aeruginosa*

Culture of *P. aeruginosa* was used for preparing a bacterial solution equivalent to 0.5 McFarland ($1.5 \times l0^8$ bacteria/mL) or OD_{600}=0.13 medium. Then the medium was left in an incubator shaker for 1h at 37°C, 250 rpm to double bacterial number to OD_{600}= 0.26 as reported previously.[311] An equal volume of the antibiotic at 1 MIC, 1/2 MIC, 1/4 MIC, and 1/8 MIC and then 25 mL of medium were added to the 5th flask (control). 25 mL of the doubled bacteria was added to all flasks to get a total volume of 50 mL solution in each flask. Flasks were then incubated at 37°C at 250 rpm. Bacteria cell density was measured spectrophotometrically at 600 nm every 1 h for 8 h intervals and then growth was measured at 24 h again. The results were taken from three separate experiments.

2.2.12 Determining antibacterial activity of liposomal azithromycin against PAO1 by MBEC Assay

Pseudomonas aeruginosa PAO1 strain was adjusted to 0.5 McFarland standards ($1.5\times l0^8$ cfu/mL) were transferred to MBEC^{TM} (CBD- Innovotech, AB, Canada) plate and incubated at 37°C for 72-96 h. Every 24 h, broth in the plate was replaced to remove the unattached bacteria from lid pegs and the plates with pegs, which were washed with PBS using another 96 welled

plate. After 96 h biofilms were harvested from the pegs by using sterile plier and suspended in tubes of 900 µL PBS. Sonication was applied to detach bacteria from the pegs for total time period of 60 sec with every 10 sec interval for vortexing the tubes. The bacteria were then treated with different concentrations of free azithromycin and liposomal azithromycin for 24 h at 37°C.

The samples of bacteria untreated with drugs were considered as control. The tubes containing peg lids with PBS was subjected to 10 fold serial dilution to reduce the concentration and 100 µL of each sample were plated on CAMH agar plates for 24 h at 37°C for determination of cfu.

2.2.13 β-Galactosidase activity assay

The AHL production levels from *P. aeruginosa* exposed to free and liposomal azithromycin at subinhibitory concentrations were tested by measuring the ability of *P. aeruginosa* AHL signaling molecules released into the supernatants to activate the production of β-galactosidase in the reporter strain *A. tumefaciens* (A136).[312] Briefly, bioassay tubes containing 4 mL of reporter strain and 1 mL of supernatant were incubated at 30°C in a water bath for a period of 5 h with rotation at 100 rpm. Following with a measurement of the bacterial cell density (as the OD_{600}) prior to the centrifugation. The supernatants were eliminated, and the pellets were suspended in an equal volume of Z buffer. The cells were then subjected to permeabilization by a solution of 200 µL of chloroform and 100 µL of 0.1% sodium dodecyl sulfate prior to the addition of 0.4 mL of o-nitro phenol-β-D-galactopyranoside (4 mg/mL in PBS). Once the yellow color was developed, 1 mL of 1 M Na_2CO_3 was supplemented to stop the reaction. The optical densities of the reaction samples were measured at 420 and 550 nm. Miller units of β-galactosidase activity was calculated by the formula $[(1{,}000 \times A_{420}) - (1.75 \times A_{550})]/$

(time × volume × A600).[251,313] Results were represented in residual activity in percentage and control used corresponds to 100%.

2.2.14 Virulence factors assays

2.2.14.1 Lipase assay

0.6 mL of filtered supernatant of bacteria, 0.6 mL of Tween® 20 in Tris buffered saline (10%), 0.1 mL of $CaCl_2$ (1 M), 1.2 mL H_2O were mixed in a 15 mL tube and incubated at 37°C for 24 h with agitation at 200 rpm (Innova 4000 Incubator Shaker; New Brunswick scientific, NJ). In the presence of lipase, tween is broken down and bound to calcium which precipitates and can be measured by turbidity (OD_{400}).[314]Lipase experiments were done three times in triplicates.

2.2.14.2 Chitinase assay

The filtered supernatants (1 mL) of bacteria were mixed with 5 mg of insoluble chitin azure, and 1 mL of PBS in a 15 mL tube. The mixture was incubated at 37°C for 24 h with agitation at 200 rpm (Innova 4000 Incubator Shaker; New Brunswick scientific, NJ). After centrifugation (16,000 x *g*) to remove insoluble chitin azure, the absorbance was measured at OD_{290}.[314]The experiments were repeated three times in triplicates.

2.2.14.3 Elastase assay

The filtered supernatants (1 mL) were mixed with 20 mg of insoluble elastin-Congo red, and 1 mL of PBS in a 15 mL tube. Elastin-Congo red is insoluble and will sediment at the bottom. The mixture was incubated at 37°C for 24 h with agitation on a shaker at 200 rpm

(Innova 4000 Incubator Shaker; New Brunswick scientific, NJ).[315] If elastase is present, it would breakdown and dissolve elastin producing a red colour. By centrifugation (16,000 x *g*), the insoluble elastin-Congo red was removed, and the absorbance was read at OD_{495}. All the elastase experiments were done three times in triplicates.

2.2.14.4 Protease assay

The filtered supernatants (200 µL) obtained from untreated or treated bacteria were added to the wells of Petri dishes (2% agar) containing 3% skim milk was incubated for 48 h at 37°C.[316] Mixed the solution to dissolve and poured 25 mL into each Petri dish, and solidified. Made holes in the agar with the head of a 1 mL pipette tip and added about 200 µL of the supernatants carefully into the hole. Incubate the dishes for 24 h at 37°C. The zones of clearance (diameter) around the holes were measured directly from the plates with the use of calipers, with three repeats and four replicates, and the experiments were done three times in triplicate.[315]

2.2.14.5 Motility studies

The motility studies were performed as mentioned by other investigators.[311] PAO1 grown overnight was diluted to 0.5 McFarland standard (1.5×10^8 cfu/mL), and 1µL of PAO1 was inoculated onto a 3-mm depth of agar plates containing a subinhibitory concentration of free or liposomal azithromycin (1/16 to 1/32 the MIC). Inoculation into the bottom of CAMH broth with agarose (1% [wt/vol]) was used for the twitching experiment, and point inoculation onto the medium with agarose (0.3% [wt/vol]) was used for swimming and swarming experiments (0.5% [wt/vol]). After 12 h of incubation at 37°C, swimming and swarming diameters were measured. The zones of twitching at the agarose-petri dish interface with the medium on the dish surface was slightly removed after 24 h of incubation at 37°C and then visualized by staining for 1

minute with the use of 1% [wt/vol] crystal violet and their diameters were then measured. All the swimming, swarming and twitching experiments were performed in three separate experiments in triplicate.

2.2.15 Determination of bacterial membrane fusion with liposomes by flow cytometry analysis

Flow cytometry has been used to evaluate the fusion of liposomes with bacteria. The liposomes were labeled with PKH2-GL with the use of a PKH2-GL labeling kit (Sigma, St. Louis, MI).[317] To study interactions between biological membranes a PHK2-GL probe was used in this experiment. In brief, freeze-dried liposomes were rehydrated with PBS and the obtained liposomal pellet was used for the labelling procedure. Labelling was done by following the manufacturer's instructions, in which 1 mL aliquot was taken and centrifuged for 30 min at 6000 x *g* under 4°C. The pellet was then resuspended in 1 mL of diluent A (Sigma, St. Louis,MI) and 8 µL of PHK2-GL was added to the pellet. The PHK2-GL liposomal solution was incubated for 5 min at room temperature with circular agitation. Following the incubation, 2 mL of bovine serum albumin at 1% (w/v) in PBS was added and the solution was further incubated for 1 min with agitation to stop the labelling reaction. Liposomes were then washed twice with PBS by centrifuging at 5000 x *g* for 15 min under 4°C to remove any free PKH. Labeling efficiency was determined by flow cytometric analysis.

The Incorporation of liposomes to bacterial cells was demonstrated in a fluorescence-activated cell sorting (FACS) analysis using the fluorescent marker PKH2-GL. PAO1 were incubated for 18 h in MH broth and a solution of 0.3 of OD (660 nm) was then prepared. The solution was centrifuged and the bacterial cell pellet was resuspended in RPMI supplemented

with 2.5% fetal calf serum for washing. The final cell pellet obtained was resuspended in RPMI supplemented with 2.5% fetal calf serum to obtain the same initial concentration. Aliquots of 18 mL were then taken and transferred to 50 mL flasks. Bacteria were incubated with 200 µL of liposomes labeled with PHK2-GL, 200 µL of PBS (negative control) and 80 µL of free PKH-2GL (positive control). PBS was then added to the flasks to complete the volume to 20 mL and the flasks were incubated at 37°C with agitation (250 rpm). Azithromycin was not encapsulated in liposomes in order to avoid bacterial cell killing. 2 mL samples were taken after 1, 5 and 10 h of incubation. Liposomes were washed with 2 mL of sucrose cushion of 21% (w/v) in PBS to eliminate free liposomes and the excess PKH2-GL. The obtained bacterial pellet was washed twice in the presence of PBS and the final cell pellet was fixed with 300 µL of 2% formaldehyde diluted in PBS for flow cytometry analysis. Samples were analyzed in duplicate and data confirmed the slight fusion of liposomes with bacterial cell membranes. The fusion data provided by PKH2-GL fluorescence shows the liposome bacterial membrane interactions.

2.2.16 Cytotoxicity test

The cell toxicity studies were done by MTT (Methyl thiazol tetrazolium) assay as mentioned previously.[306]In this study, human lung carcinoma epithelial cell line (A549; ATCC, Manassas, USA) was used for the cell toxicity assay. The lung cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). For the MTT assay, the cells were implanted in to 96-well plates at a density of 5 x 10^3 cells/well for sub-confluency. The media was eliminated from the wells and replaced with media containing the azithromycin or liposomal azithromycin. The untreated cells containing media alone were considered as controls. Further, plates were subjected to incubation for 24 h in 5% CO_2 at 37°C. After 24 h of exposure, the well was washed once with entire medium and once with DPBS, and replaced with fresh media. MTT

dye was supplemented to each well and the plates were incubated in the dark for 4 h at 37°C and MTT lysis buffer was then added to each well of the plate. The well plates were further incubated in the dark overnight at 37°C, before taking the measurement at 590 nm spectrophotometrically. Cytotoxicity studies were illustration of three separate experiments, performed in triplicates.

2.2.17 Hemolytic test

To further invesitigate the toxicity of liposomal azithromycin hemolytic test was done by erythrocyte hemolysis assay as described in previous studies.[318,319] The human erythrocytes were pooled and subjected to centrifugation (700 x *g* for 10 minutes at 4°C); they were washed twice by centrifugation (700 x *g* for 10 minutes at 4°C) with ten times the volume of an ice cold buffer. Later, the washed erythrocytes were re-suspended in twenty times their quantity of fresh, ice cold buffer. In order to perform the hemolysis assay, a 3% erythrocyte solution was prepared by suspending the required quantity of erythrocytes into a hemolysis buffer solution. In a shaking water bath, erythrocytes were incubated under gentle agitation at 37°C with empty or liposomes containing azithromycin up to 24 h. After incubation, erythrocyte samples were removed and subjected to centrifugation (1000 x *g* for 10 min at 4°C).

The obtained supernatant was discarded and was centrifuged (15000 x *g* for 15 min at 4°C). In the next step, supernatant was removed and extent of hemoglobin release was measured spectrophotometrically at 540 nm. The 3% erythrocytes which stand for 0% hemolysis and red blood cells with 1% Triton X-100 indicating 100% red blood cell lysis were considered as controls in this study. The hemolysis buffer alone was considered as blank for the measurement.

For determining the percentage of hemolysis, blank of hemolysis buffer was subtracted from all values obtained and it is estimated by the equation:

$$\% \text{ Hemolysis} = (Abs._{Sample} - Abs._{Control\ 0\%}) / (Abs._{100\%\ Lysis}) \times 100$$

2.2.18 Data analysis

The data presented as mean ± standard errors of the mean (S.E.M.) of three independent experiments. The comparisons of groups were done by one-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test by using InStat 3 from Graph Pad prism (GraphPad Software Inc., Version 5.0) followed by a post-hocs using t-tests. Probability values of *$*P<0.05$, **$P<0.01$ and ***$P<0.001$* are considered as statistically significant.

2.3 Results

2.3.1 Liposomal azithromycin characterisations and stability

The mean particle size of liposomes was 406.07 $\pm$ 45 (nm) and polydispersity index (PI) was 0.3 ± 0.03. The encapsulation efficiency of liposomal azithromycin was 23.8 ± 0.2 % and concentration of drug entrapped in the liposomes was 0.51 ± 0.004 mg/mL**.**

The DSC curves of pure DPPC, physical mixture 1 (DPPC and Cholesterol) and physical mixture 2 (DPPC, Cholesterol and azithromycin) are showed in (Figure S1). The melting point (Tm) for pure DPPC was found to be 41.98℃. The Tm for physical mixture-1 (DPPC and Cholesterol) and physical mixture-2 (DPPC, Cholesterol and Azithromycin) were found to be 41.96℃ and 41.98℃ respectively. The melting transition of liposomal azithromycin was found to be 40.91℃, which was slightly reduced compare to the physical mixtures 1 and 2.

The stability studies of liposomal azithromycin were performed in PBS at 4°C and 37°C (Figure S2). The study was performed for a period of 48 h. The liposomal azithromycin stored at 4°C was more stable compared to that stored at 37°C. Retention of liposomal azithromycin at 4°C was 95%, whereas at 37°C, the retention was 76.3% at the end of 48 h incubation study period. At 2 h of incubation from the initial period liposomal azithromycin retention was decreased to 94.6% and 80.3% for 4°C and 37°C respectively.

2.3.2 Determination of the minimum inhibitory concentrations and minimum bactericidal concentrations

The MIC and MBC values of free and liposomal azithromycin against *P. aeruginosa* are presented (Table 4). The experiments were performed on both mucoid and non-mucoid of *P. aeruginosa* strains. The MIC values of liposomal azithromycin against both mucoid and non-mucoid *P. aeruginosa* strains were significantly lower than those of free azithromycin. For example, the MIC value of *P. aeruginosa* strain (PAO1) was 16 mg/L for liposomal azithromycin and 128 mg/L for free azithromycin. The MBC value of PAO1 was 32 mg/L (42.7 μM) for liposomal azithromycin whereas, the MBC value for free azithromycin was 256 mg/L (341.8 μM).

2.3.3 Effect of subinhibitory concentrations of free and liposomal azithromycin on the growth of *P. aeruginosa*

Among all the subinhibitory concentrations (1/2 the MIC to 1/16 the MIC), 1/16 the MIC did not inhibit the growth of bacteria as shown in (Figure 1). Liposomal azithromycin at 1/2, 1/4 and 1/8 the MIC exhibited a significant reduction in bacterial growth ($P < 0.001$) compared to control after 2 h of incubation and throughout the study, thereby liposomal azithromycin and free

azithromycin at 1/2, 1/4 and 1/8 the MIC considered as inhibitory concentration. For this reason 1/16 the MIC and 1/32 the MIC have been selected to show the effect of subinhibitory concentrations of free and liposomal azithromycin on the bacterial virulence factors, motility and quorum sensing molecules reduction experiments.

2.3.4 Minimum biofilm eradication concentration assay

P. aeruginosa biofilm exposed to free and liposomal azithromycin at concentrations between 8 mg/L to 1024 mg/L (Figure 2). Liposomal and free formulations were able to eradicate bacteria completely at concentrations of 1024 mg/L and 512 mg/L. However, liposomal azithromycin formulation at concentrations 256 to 8 mg/L significantly reduced bacterial counts compare to control and of free azithromycin $P<0.001$. For example, Liposomal formulation at 8 mg/L was more significant in reducing bacterial count compared to 32 mg/L of free azithromycin ($\log_{10}$ 5.6 *versus* $\log_{10}$ 6.3) ($P<0.001$). Furthermore, Liposomal formulation significantly reduced bacterial count at concentration two times lower than the free formulation (128 *versus* 256 mg/L) ($\log_{10}$ 3.7 *versus* $\log_{10}$ 4.4) ($P<0.001$).

2.3.5 Quorum sensing molecules reduction

PAO1 strain was exposed to free azithromycin and liposomal azithromycin at 1/16 the MIC and 1/32 the MIC (Figure 3). The decreased levels of Acyl homoserine lactones (AHL) from β-galactosidase activity levels indicated a reduced level of AHL signaling molecules. Liposomal azithromycin reduced QS molecules compared to control and free formulation. For example, liposomal azithromycin at concentration 1/16 MIC exhibited a significant reduction effect on QS compared to 1/16 MIC of free azithromycin and control ($P<0.001$). Furthermore, liposomal azithromycin at concentration 1/32 MIC resulted in a significant reduction compared

to 1/32 MIC of free azithromycin and control (*P<0.01*). However, liposomal formulation at 1/16 the MIC resulted in significant reduction compared to 1/32 the MIC of free formulation (*P<0.001*).

2.3.6 Effect of liposomal azithromycin on bacterial virulence factors

The virulence factors like lipase, chitinase, elastase and protease levels of *P. aeruginosa* PAO1 strain when exposed to free and liposomal azithromycin at 1/16 the MIC and 1/32 the MIC were examined (Figure 4). Liposomal azithromycin at 1/32 the MIC had a slight effect on the lipase production compared to control, whereas free azithromycin had no noticeable effect on lipase production (Figure 4a). Furthermore, the liposomal formulation at 1/16 the MIC reduced the lipase activity significantly compared to control (*P<0.01*). For chitinase activity, liposomal azithromycin at 1/32 the MIC reduced the chitinase production compared the control (Figure 4b). Furthermore, liposomal formulation reduced the chitinase production significantly at concentration 1/16 the MIC compared to control (*P<0.05*). For elastase, free azithromycin did not reduce the elastase activity signifacntly compared to control, whereas liposomal azithromycin at 1/16 and 1/32 the MIC reduced elastase production significantly compared to control (*P<0.01*) and (*P<0.05*), respectivly (Figure 4c). Furthermore, liposomal formulation was more significant in reducing elastase activity compared to free formulation at 1/16 and 1/32 the MIC (*P<0.05*). The protease activity was measured by the halo zones formed in the petri dishes containing skim milk (Figure 4d). Liposomal azithromycin at concentration of 1/16 the MIC significantly reduced the protease production compared to the free azithromycin and control (*P<0.001).*

2.3.7 Effect of liposomal azithromycin on bacterial motility

The *P. aeruginosa* motility patterns including twitching, swarming and swimming were examined in the presence of the free and liposomal azithromycin at 1/16 the MIC and 1/32 the MIC (Figure 5). For motility including twitching, swarming and swimming, the liposomal azithromycin at 1/16 the MIC significantly reduced all three motility patterns compared to 1/16 MIC of free azithromycin (*P<0.001)*. Similarly, liposomal azithromycin at concentration 1/32 MIC resulted in a significant reduction of different motility patterns compared to 1/32 MIC of free azithromycin and control (*P<0.001)*. Significant results for liposomal azithromycin were obtained in all the patterns in comparision with the controls and free azithromycin (*P<0.001).*

2.3.8 Liposome-bacterial interactions by flow cytometry

Labelled liposomes without antibiotics were utilized to avoid bacterial death due to antibiotics. The fusion of Liposomal bilayer-PKH2-GL with PAO1 reached 9.9% within 1 hour and reached a maximum of 14.6% after 5 h of contact showing strengthening of liposomes fusion with bacteria. The fusion signal reached 12.3% at 10 h of contact before the fluorescent signals began declining (Figure 6). A negative panel in the figure indicates the peak of bacterial florescence without label, and positive panel were in the range of 50-65% for the duration of the experiments. The positive panel assured that PKH2-GL was compatible with the bacterial membrane.

2.3.9 Cytotoxicity and hemolytic tests

No cytotoxic effect on A459 cells was observed after incubation with liposomal antibiotic (100%). However, 90% of the cells were viable in presence of free antibiotic. No significant

hemolysis was observed with empty or liposomes containing azithromycin, the hemolytic activities were less than 1%.

2.4 Discussion

The present study focused on the preparation of novel liposomal azithromycin formulation by DRV method.[236] Earlier study reported that preparation of liposomes by DRV method resulted in higher encapsulation efficiency of macrolide antibiotics with more stability.[193] The particle size and polydispersity index (PI) of the liposome formulations is one of the important parameter in the characterisations that represents the homogeneity of the liposomes and also for determining stability for long-term.[320] In this study, the obtained results for particle size and polydispersity index of liposomal azithromycin formulation were homogenous in nature indicating the long-term stability.[166]

The stability studies are another important considerable parameter while developing a novel liposomal formulation. Considering this parameter we performed stability measurement for liposomal formulation at different environmental conditions (4°C and 37°C) in PBS for a period of 48 h. The obtained data reveals that the retention of liposomal azithromycin at 4°C was 95% and at 37°C it was 76.3% concluding that the liposomal azithromycin was more stable at 4°C in comparison to 37°C, this is mainly due to the increase in the acyl chain length of lipid constituents there by increasing the stability of formulations containing DPPC at higher transition temperatures.[173] The incorporation of cholesterol during the preparation of liposomes may enhance the stability by reducing the bilayer permeability of the liposomal membrane, which leads to hike of drug retention at higher temperatures.[173,321] After 2 h of incubation from

the initial period liposomal azithromycin retention was decreased for both 4°C and 37°C may be due to the structural rearrangement of lipid membranes and later to become consistent.[322]

DSC is an analytical technique and it is used for characterising the melting and crystallization properties of crystalline materials.[323] The data obtained from DSC studies shows that slight reduction in the melting transition of liposomal azithromycin when compared to physical mixtures (DPPC, Cholesterol and Azithromycin) results from the interactions between lipid material and azithromycin. The intercalated drug molecules might disrupt hydrogen bonds of liposomes which lead to change in the structural arrangement of lipid layers, thereby reducing the melting transition of the azithromycin loaded liposomes.[310,324,325] These DSC profile also provide a molecular understanding of the interaction between a macrolide antibiotic and phospholipids, which could improve understanding of azithromycin–cell interaction.[81] The molecular interaction of azithromycin and DPPC reveals that phase transition of DPPC is strongly decreased due to the increase of gap between hydrophobic chains after incorporation of azithromycin into the lipid bilayers.[174,326]

The results from our data demonstrate that the encapsulation of azithromycin into liposomes shows more effective anti-microbial activity than the free one against *P. aeruginosa*. The MIC and MBC values of liposomal azithromycin were significantly 8-fold lower than the free azithromycin indicating lower MIC values. Similar observations were reported by other investigators, showing that the liposome loaded azithromycin effect was bactericidal against *P. aeruginosa* than free drug due to the increased interaction of liposomal membrane with bacterial cell membrane were the drug was delivered directly in the cytoplasm of the bacteria.

Quorum sensing is the cell-to-cell communication that plays an important role in the formation of biofilm and virulence factors production which are contributed to resistance to antibiotics.[75,327,328]Liposomal azithromycin formulation effectively reduced the production of quorum sensing molecules at subinhibitory concentrations. The underlying mechanism involved may be due to the suppression of *las* and *rhl* in the quorum sensing system by azithromycin.[329] The liposomal azithromycin reduced QS more significantly when compared to free azithromycin; the possible reason might be due to the increased reduction of chemical signaling molecules levels by enhancing antibiotic intake into the cell to fuse with the ribosomes resulting in the down regulation of QS genes. The use of liposomes as a drug delivery system may increase the efficacy of drugs and the penetration of drugs into the biofilms by longer contact with the bacterial biofilm.[244,246]

The liposomal azithromycin at subinhibitory concentrations reduced the production of virulence factors including lipase, chitinase, elastase and protease. Earlier studies showed that azithromycin reduced different virulence factors at subinhibitory concentrations,[328] by interference with protein synthesis resulting in the decreased production of virulence factors.[330] Earlier studies showed that liposomes promotes the consumption of encapsulated antibiotic there by increase in the down regulation of quorum sensing and virulence factor gene expression or also might be due to the reduction of post-transcription synthesis.[244,251] A similar kind of result demonstrated that clarithromycin loaded liposome reduced different virulence factors production.[236]

Many studies reported that macrolide antibiotics at subinhibitory concentrations inhibited the bacterial motility, mainly by interfering with the gene expression and affecting the motility components of the bacteria.[331,332] We determined the effect of our novel liposomal azithromycin

formulation by reducing the motility patterns of the bacteria like twitching, swarming and swimming. It may have occurred due to the improved delivery of the drug into the bacterial cell membranes by liposome loaded drugs, thereby interfering with the motility components like flagella and pili of the bacteria as direct delivery of antibiotic in to the cytoplasm of bacteria there by inhibiting the flagella and type IV pilus activities.[193,332]

In order to investigate the bacterial cell membrane fusion with liposomes we performed FACS analysis. The bacterial fusion was assessed by integration of phospholipid - PKH2-GL in bacterial cells. The main characteristic feature of PKH2-GL is used to investigate cell mobility and interactions.[333] The probe facilitates tracking liposome interaction with the bacterial cell membrane by FACS analysis.[334] A study showed that the integration of PKH2-GL-labelled liposomes with bacterial membranes indicates a direct incorporation of phospholipids into the bacterial cell membranes because the probe inserts its aliphatic carbon tails into membranes as well as the dissociation of the probe from the liposomes to the bacterial membrane is due to the strong hydrophobic nature of the probe.[131] The incorporation of PKH2-GL liposomes into bacterial membranes confirms the integration of liposomes with bacterial cell membranes. Similar results were reported earlier by other investigators supporting this study, which showed the enhanced bactericidal effect of liposomal formulation was due to fusion interaction of liposomal membrane with bacterial cell membrane.[252,317]

The data from the MTT assay shows that the liposomal azithromycin has no significant cytotoxic effect on A549 cells. Exposure of A549 human lung cells to liposomal azithromycin reduced its toxicity. In supporting this hypothesis, earlier research showed that the different antibiotics loaded liposomal formulations reduced its toxic effects on A549 human lung cells.[306] For the primary assessment of phospholipids toxicity of liposomes, blood is used for better

understanding of the toxicity screening of liposomes.[319,335] In order to investigate the toxicity of liposomal azithromycin the hemolytic test was performed by erythrocyte hemolysis assay. The data shows that no reported hemolytic activity with empty or liposomes containing azithromycin suggesting that liposomes are non-toxic on human erythrocytes.

In conclusion, liposomal azithromycin formulation exhibited a significant reduction on *P. aeruginosa* bactericidal concentration, bacterial counts within biofilms, QS molecules, virulence factors and motility. Liposomal azithromycin showed no toxicity *in vitro* and could improve *P. aeruginosa* infection treatment in CF patients. These data indicate that the liposomal formulation could be a useful therapy to enhance the safety and efficacy of azithromycin against *P. aeruginosa* lung infection in cystic fibrosis patients.

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Table and Figure legends:

Figure 1: Effect of subinhibitory concentrations of free and liposomal azithromycin on the growth of PA01 at 1/2 the MIC (a), 1/4 the MIC (b), 1/8 the MIC (c), 1/16 the MIC (d). All the experiments were done in triplicate with means standard error of measurement.

Figure 2: Effect of free and liposomal azithromycin on *P. aeruginosa* PAO1 biofilm by MBEC assay. Free and liposomal azithromycin formulations were injected to the biofilm of concentrations from 8 mg/L to 1024 mg/L. The controls were untreated biofilm and data represent three independent experiments in triplicate and are shown as means ± SEM. *P* values were considered significant compared with the control: ****P<0.001*.

Figure 3: QS molecules production is measured by β-galactosidase activity. In the presence of free and liposomal azithromycin at 1/16 the MIC and 1/32 the MIC, *P. aeruginosa* PAO1 strain was exposed to free and liposomal azithromycin. Then the supernatants were collected and incubated with the reporter strain *Agrobacterium tumefaciens* (A136). The Miller unit is used to measure the β-Galactosidase activities. Each bar represents the mean ± S.E.M. of three independent experiments. *P* values were considered significant when compared with the control and between groups: ***, *P<0.001* and **, *P<0.01*.

Figure 4: Effects of subinhibitory concentrations (1/16 the MIC and 1/32 the MIC) of free an liposomal azithromycin on virulence factors production of PA01. (a) Lipase. (b) Chitinase. (c) Elastase. (d) Protease. The results represented the mean ±SEM in triplicates of three independent experiments. The results were normalized by dividing the average absorbance of the virulence factor assays over the OD_{600} (bacterial density) at 24 h for lipase, chitinase, and

elastase experiments. *P* values were considered significant compared with the control: ***, *P<0.001; **, P<0.01; *, P<0.05.*

Figure 5: Effect of a subinhibitory concentration of liposomal azithromycin on *P. aeruginosa* motility. The motility was examined at free and liposomal azithromycin at 1/16 the MIC and 1/32 the MIC. Twitching (1% agarose [wt/vol]) (a), swarming (0.5% agarose [wt/vol]) (b), and swimming (0.3% agarose [wt/vol]) (c) *P* values were considered significant compared with the control and between groups: ***, *P<0.001* and **, *P<0.01.*

Figure 6: Flow cytometry histograms- fusion (%) of labelled liposome-PKH2-GL with PAO1 at 1, 5 and 10 h intervals.

Table 4: Antimicrobial activities of both free and liposomal azithromycin.

	Free AZM(µg/ml)		**Lipo AZM(µg/ml)**	
***P. aeruginosa* strains**	**MIC**	**MBC**	**MIC**	**MBC**
PAO1 (M)	**128**	**256**	**16**	**32**
PA-13641-1 (NM)	**64**	**128**	**8**	**16**
PA-13641-2 (M)	**512**	**1024**	**32**	**64**
PA-48912-1(NM)	**128**	**256**	**16**	**32**
PA-48912-2(NM)	**256**	**512**	**32**	**32**
PA-M 13640(M)	**512**	**1024**	**32**	**64**
PA-1(NM)	**512**	**1024**	**128**	**256**
PA-5(NM)	**256**	**512**	**32**	**64**
PA-3(NM)	**512**	**1024**	**64**	**128**
PA-7(NM)	**256**	**512**	**16**	**32**

(M)- Mucoid (NM)- Non mucoid

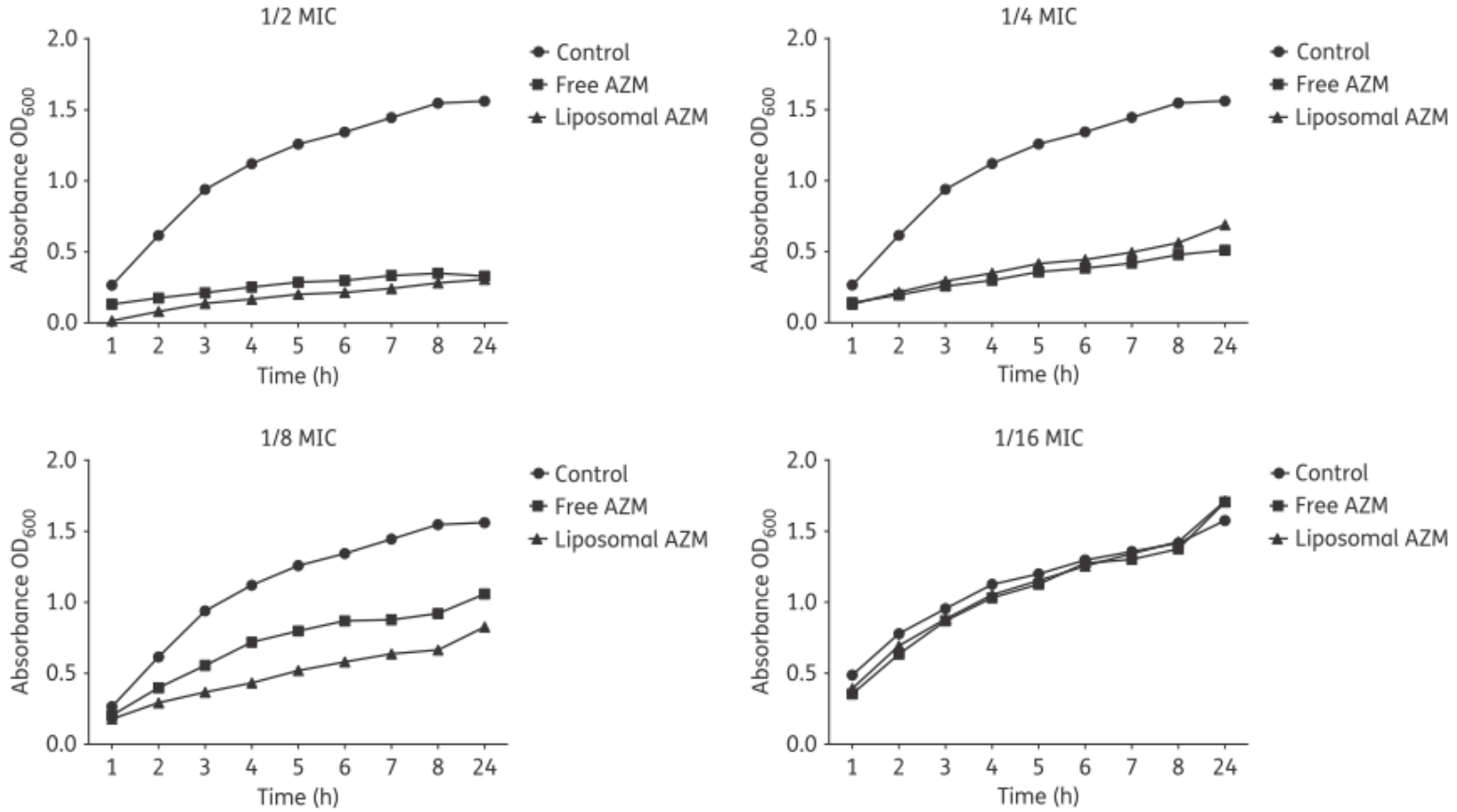


Figure 1: Effect of subinhibitory concentrations of free and liposomal azithromycin on the growth of PA01.

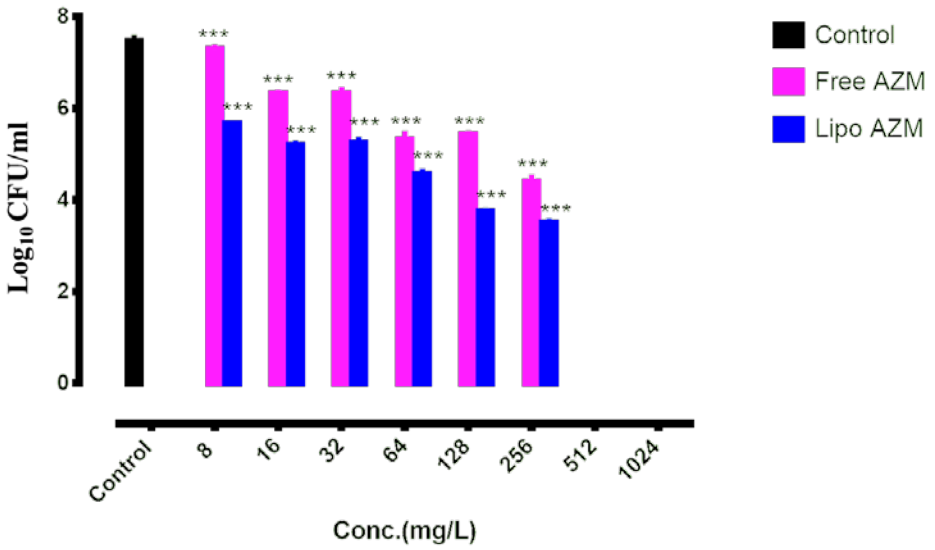


Figure 2: Effect of free and liposomal azithromycin on *P. aeruginosa* PAO1 biofilm by MBEC assay.

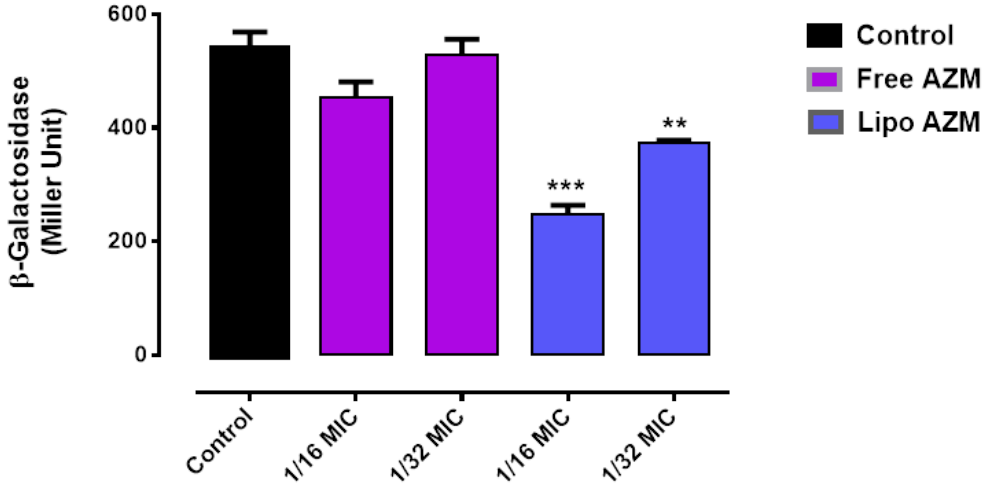


Figure 3: QS molecules production is measured by β-galactosidase activity

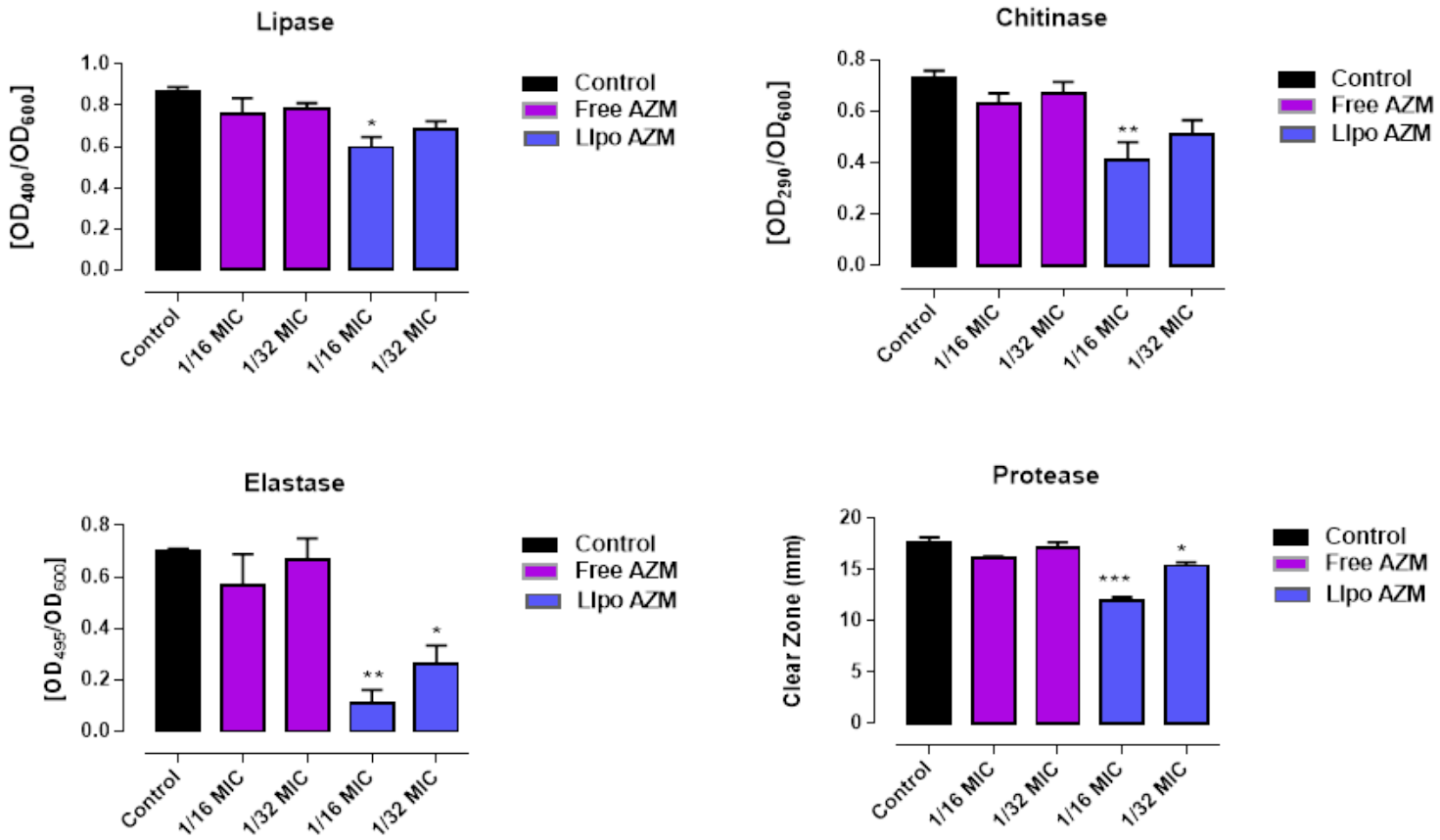


Figure 4: Effects of subinhibitory concentrations of free and liposomal azithromycin on virulence factors production of PA01.

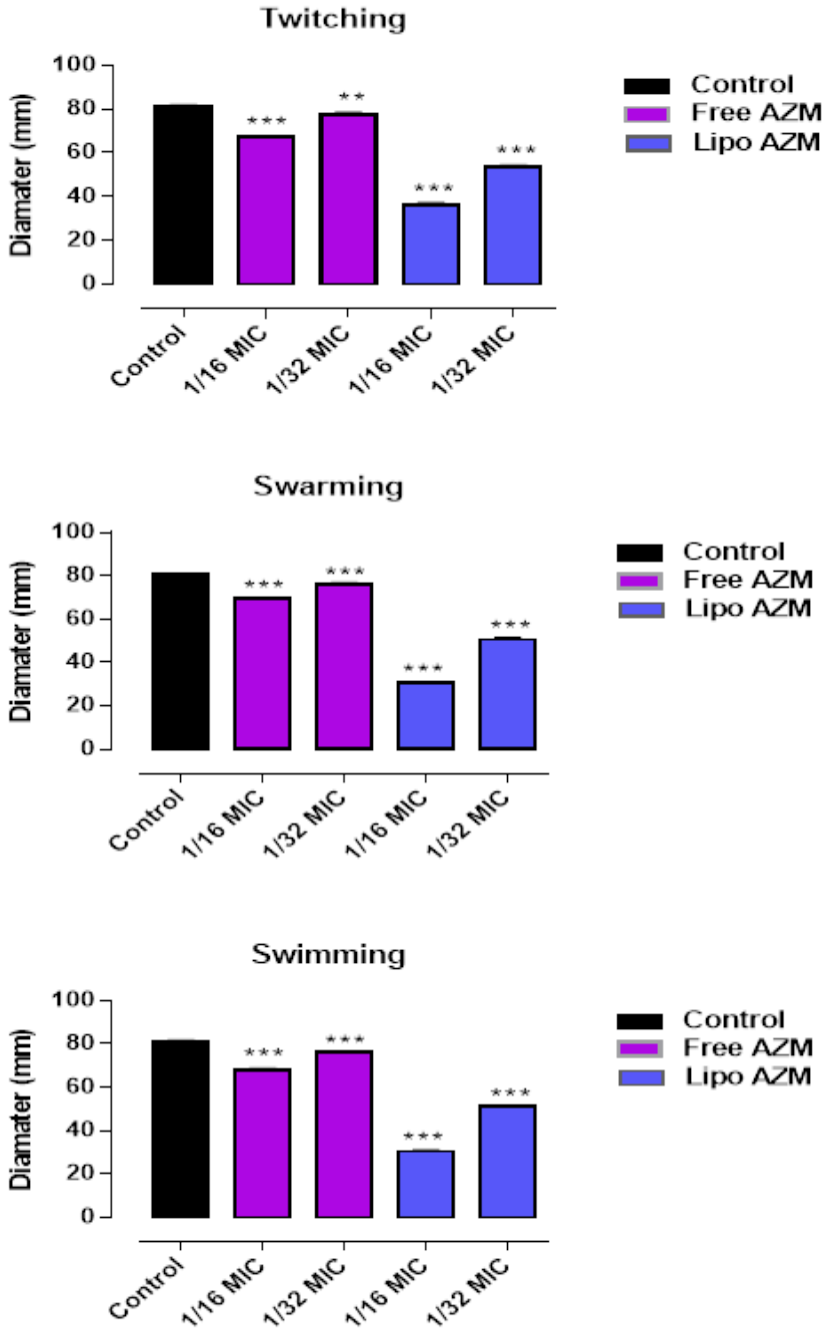


Figure 5: Effect of a subinhibitory concentration of liposomal azithromycin on *P. aeruginosa* motility.

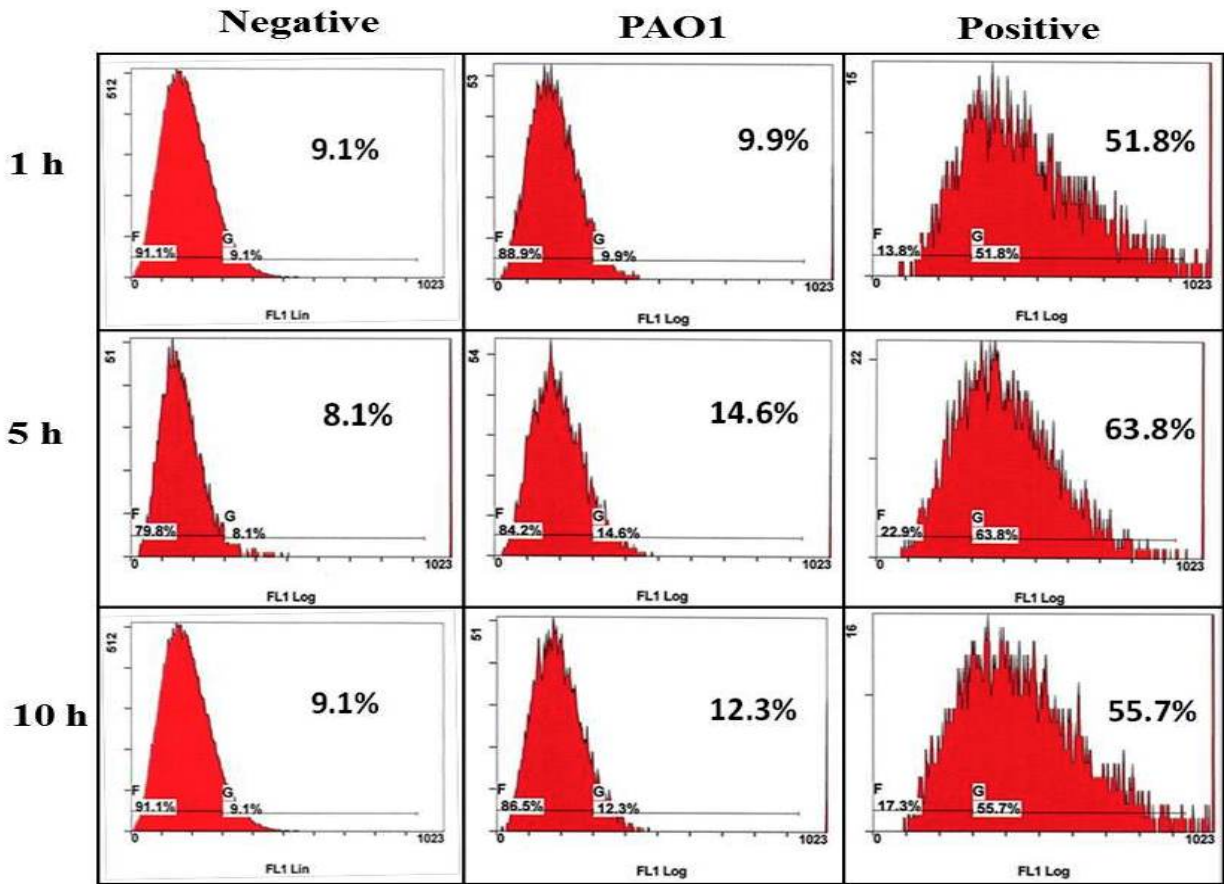


Figure 6: Flow cytometry histograms- fusion (%) of labelled liposome-PKH2-GL with PAO1

Supplementary figure legends:

Figure S1: DSC profile. PM-1: Physical Mixture-1 (DPPC, Cholesterol), PM-2: Physical Mixture-2 (DPPC, Cholesterol and Azithromycin) Dashed dot line for liposomal azithromycin, Dotted line for PM-2, Dashed line for PM-1, Black line for DPPC.

Figure S2: Stability profile of liposomal azithromycin.

Figure S1:

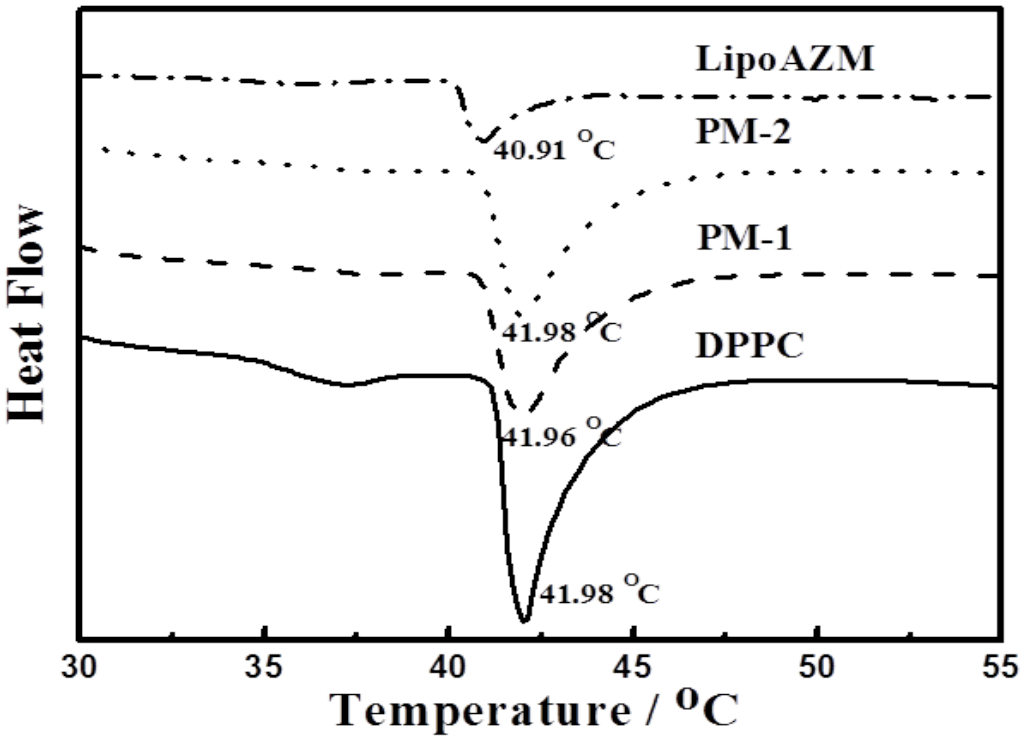
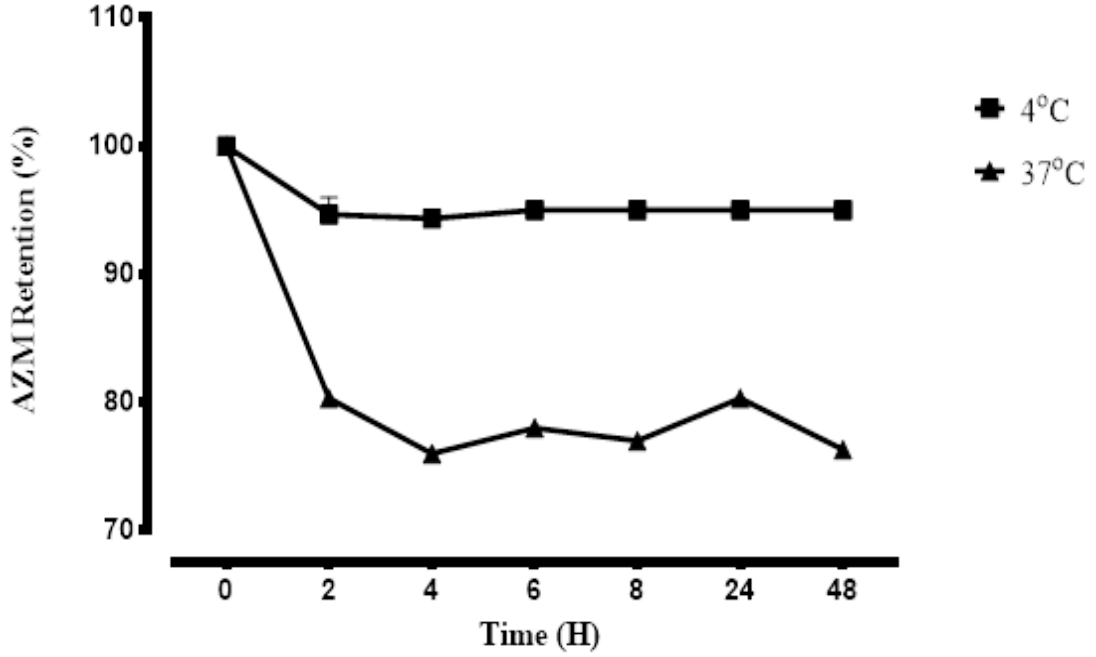


Figure S2:



Chapter 3

3 Conclusion and future directions

P. aeruginosa is the most significant pathogen involved in the pulmonary infection of CF. The effective use of antibiotics such as aminoglycosides, beta-lactams, fluoroquinolones and macrolides are found to be useful in the infection control of *P. aeruginosa*, decreased morbidity and increased life expectancy of CF individuals. The encapsulation of antibiotics in the liposomes have shown increased antibacterial activity against *P. aeruginosa* and reduced toxicity.

The main objective of this research is to develop a novel liposomal azithromycin formulation and evaluate its antimicrobial effects against *P. aeruginosa*. We developed a novel liposomal formulation loaded with azithromycin prepared by dehydration-rehydration vesicle (DRV) method and related characterizations were performed. Liposomal azithromycin activity against biofilm forming *P.aeruginosa* was assessed using Calgary biofilm device (CBD). The effect of sub inhibitory concentrations of liposomal azithromycin on bacterial virulence factors and motility studies was performed on *P.aeruginosa* strains. In addition, the bacteria and liposome interactions were studied and toxicities were evaluated *in vitro.*

Our data indicated that liposomal azithromycin has significant encapsulation efficiency and long-term stability. The MIC and MBC values were lower compare to the free azithromycin, indicating an effective antimicrobial activity for liposomal azithromycin. Considerably, the liposomal azithromycin reduced bacteria formation in the biofilm. At sub-inhibitory concentrations, the QS as well as virulence factors such as lipase, chitinase, elastase and protease production was mitigated. In addition, the different patterns of bacterial motilities (swimming, swarming and twitching) were restricted. Flow cytometry analysis showed remarkable

interactions between liposomes and bacterial membrane. Neither lung cell toxicity nor significant erythrocyte lysis was observed by liposomal azithromycin.

Based on our in vitro data, our liposomal azithromycin formulation could be an effective and therapy against *P. aeruginosa* lung infection in CF patients. In conclusion, liposomal drug delivery systems are result-driven and provide considerable option for treatment of chronic lung infections.

In the future, this research could be carried forward to evaluate efficacy in the *P. aeruginosa* infected animal models. It would also be remarkable to explore the mechanism of action of inhibiting virulence factors and QS molecules by liposomal azithromycin.

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